REMARKS

I. Claims under Consideration

Claims 5, 6, 8, 10, 12, 14, 15, and 17-19 are pending. The restriction requirement set forth in the prior Office Action has been withdrawn and all pending claims have been examined on the merits. Claims 14, 15, 18, and 19 stand rejected under 35 U.S.C. § 112, second paragraph. Claims 5-6, 8, 10, 12, 14, 15, and 17-19 stand rejected under § 112, first paragraph. Claims 5, 6, 8, 10, 13, 15, and 17 stand rejected under 35 U.S.C. § 102(e). Claims 5, 6, 8, 10, 12, 14, 15, and 17-19 were provisionally rejected under the judicially created doctrine of obviousness-type double patenting. Each of these rejections is addressed as follows.

II. Rejections Under 35 U.S.C. § 112, Second Paragraph

Claims 14, 15, 18, and 19 stand rejected under 35 U.S.C. § 112, second paragraph, as indefinite. In particular, the Office objects to the phrase "vector system" as being ambiguous and indefinite. More specifically, the Office objects to the term "system" as implying "a group of interacting, interrelated or interdependent elements forming a complex whole" or implying "additional steps, processes, elements, or components, which remain undefined and indefinite."

As mandated by the definiteness requirement of 35 U.S.C. § 112, second paragraph, a specification shall include claims "particularly pointing out and distinctly

claiming the subject matter which the applicant regards as his invention" (emphasis added). Determining whether a claim is definite requires an analysis of "whether one skilled in the art would understand the bounds of the claim when read in light of the specification . . . . If the claims read in light of the specification reasonably apprise those skilled in the art of the scope of the invention, § 112 demands no more." *Miles Lab., Inc. v. Shandon, Inc.*, 997 F.2d 870, 875, 27 USPQ2d 1123, 1126 (Fed. Cir. 1993).

In this case, applicants' written description of the specification is sufficient to inform one skilled in the art of the meaning of the claim language "vector system." It explicitly defines a "vector system" as "multiple vector molecules comprising a combination of a vector containing the fusion protein-coding DNA and a vector containing the exogenous gene, for example, a binary vector system" (see page 6, second paragraph). In other words, in the context of the present invention, a vector system comprises multiple independent yet interrelated vector molecules, at least one comprising a fusion protein and another comprising an exogenous gene. Such a vector system of multiple vector molecules is usually introduced into a cell by co-transformation. Thus, the specification amply delimits and defines the term "vector system" such that one skilled in the art would be reasonably apprised of the metes and bounds of the pending claims. Accordingly, applicants request reconsideration and withdrawal of the rejections in light of the remarks set forth above.

Claim 15 is also rejected as depending from one or more canceled claims. In view of the present claim amendment, this rejection moot.

III. Rejections Under 35 U.S.C. § 112, First Paragraph (Written Description)

The Office asserts that the claims 5-6, 8, 10, 12, 14, 15, and 17-19 are unpatentable under § 112, first paragraph, because they lack an adequate written description. According to the Office, the claims are drawn to a genus (any cytokine receptor, any portion thereof) that encompasses a vast number of structures, each with the correlative function of inducing proliferation of any cell. The Office concludes that "[g]iven the enormous breadth of the cell-proliferation cytokine receptor variants encompassed by the rejected claims and given the limited description from the instant specification of such variants, the skilled artisan would not have been able to envision a sufficient number of specific embodiments to describe the broadly claimed genus of cell proliferation cytokine receptor domain variants."

Applicants first note that *Lilly* makes clear that the written description of a genus of DNA may be achieved by a "recitation of structural features common to members of the genus." *Regents of the Univ. of California v. Eli Lilly and Co.*, 119 F.3d 1559, 1569, 43 USPQ2d 1398, 1406 (Fed. Cir. 1997). Applicants' specification, for example, in its description of the vectors which include genes that encode polypeptides and their shared, characteristic structural features satisfies this standard. Moreover, there is no question

that the present specification would certainly indicate to one of ordinary skill in the art that virtually any gene encoding a polypeptide that includes an amino acid sequence in which a portion of the G-CSF extracellular domain has been deleted (claim 5) or a polypeptide that includes a cytokine receptor (or a proliferation-inducing part thereof) that imparts proliferation activity to a cell (claim 8) is useful in the claimed methods.

Further, in response to the Office's assertion that a functional limitation cannot be used to limit the claims because "applicants only recite the claimed domains by functional means without any disclosure as between the limitless number of structures and correlative function," applicants respectfully assert that further characterization of the disclosed variants, is not necessary to distinguish the claimed fusion proteins from other proteins. As stated in the Written Description Guidelines (66 FR 1106),

> [f]actors to be considered in determining whether there is sufficient evidence of possession include the level of skill and knowledge in the art, partial structure, physical and/or chemical properties, functional characteristics alone or coupled with a known or disclosed correlation between structure and function, and the method of making the claimed invention. Disclosure of any combination of such identifying characteristics that distinguish the claimed invention from other materials and would lead one of skill in the art to the conclusion that the applicant was in possession of the claimed species is sufficient.

The claimed fusion proteins are clearly distinguished from other proteins by both the structural characteristic and by the specific functional characteristic of inducing cell proliferation. Based on applicants' disclosure of these properties and routine assays for determining whether a particular protein has these properties, one skilled in the art would

appreciate that applicants were in possession of the claimed invention. As clear distinguishing characteristics that are shared by the claimed fusion proteins are disclosed in appellants' specification, this rejection should be withdrawn.

On the issue of cytokine receptor "variants," more particularly cytokine receptor "variants" having one or more amino acid substitutions or mutations (i.e., sequence mutants), applicants note that that the instant claims are not directed to sequence mutants. Rather, the claims encompass a cytokine receptor or a portion thereof that induces proliferation (i.e., protein truncations that include the proliferation inducing domain). Unlike sequence mutants, the number of functional truncation "variants" for a particular cytokine is neither endless nor incalculable, particularly since cytokine receptors are so well studied that the proliferation-inducing domain is either known in the art or easily recognizable. Thus, applicants emphasize that the instant claims do not encompass a "vast number" of "widely divergent" cytokine receptor "mutants." Rather, the claims include either an amino acid sequence in which a portion of the G-CSF extracellular domain has been deleted (claim 5) or a polypeptide that includes a cytokine receptor (or a proliferation-inducing part thereof) that imparts proliferation activity to a cell (claim 8). Accordingly, applicants submit that this "genus" is neither vast nor widely divergent.

Applicants also point out that, in this case, the cytokine receptor "variants" encompassed by the claims are substantially similar (i.e., all include, at a minimum, the proliferation inducing domain), particularly given the fact that in terms of the

proliferation-inducing domain, little variation exists across species. Specifically, as noted in applicants' previous reply, with respect to the proliferation-inducing domain, no substantial variation exists within the family of cytokine receptors. The membrane-proximal domain that includes box1/box2 motif is well-conserved among cytokine receptor family members. (See Ihle et al. and Murakami et al. cited in the previous response.) This region is vital for cell proliferation signal transduction in a variety of cytokine receptors including, but not limited to, IL-6R, IL-2R, EPO, G-CSF, and c-MplR. Accordingly, one skilled in the art could not only routinely identify which cytokines induce proliferation upon dimerization but could also routinely envision the proliferation-inducing domain of such a cytokine. Accordingly, a limited number of representative species indeed suffices to demonstrate possession of the claimed "genus."

Further, with respect to the issue that applicants have not described a representative number of species of the claimed genus, applicants point out that "[r]epresentative examples are not required by the statute and are not an end in themselves." *In re Robins,* 429 F.2d 452,457, 166 U.S.P.Q. 552, 555 (C.C.P.A. 1970). Rather, applicants' specification "must 'convey clearly' to those skilled in the art to whom it is addressed ... the information that [the inventor] has invented the specific subject matter later claimed." *Martin v. Mayer,* 853 F.2d 500, 505, 3 U.S.P.Q.2d 1333, 1337 (Fed. Cir. 1987). Again, as noted above, applicants' specification clearly meets this standard.

The Office further suggests that unpredictability in the field of biotechnical inventions prohibits an applicant from claiming a genus when only a limited number of species are described. However, as noted in the previous response and above, at the time of filing, cytokine receptors were so well studied that their functions and structures were known in the art. In fact, in most cases, a mere review of the literature would be sufficient to distinguish those cytokine receptors predicted to be operable in the context of the claimed fusion protein from those that are not.

For example, the "Cytokines Web", found on the internet at http://cmbi.bjmu.edu.cn/cmbidata/cgf/CGF_Database/cytweb (copyright 1996 to present), provides scientific information about cytokines and their receptors, including 3-D structural information and topological and evolutionary relationships. Similarly, the "Cytokine Online Pathfinder Encyclopedia" (COPE), found on the internet at http://www.copewithcytokines.de (copyright 1997 to present), provides a continuously updated, searchable index of all known cytokines, including information regarding their receptors and associated biological activities. Thus, with only a modicum of research, one skilled in the art could routinely and predictably identify a priori which cytokine receptors would be expected to induce proliferation as a result of receptor dimerization.

It is important to note that most cytokine receptors share significant characteristics recognized by those skilled in the art. For example, signal transduction for most if not all cytokine receptors, particularly those of Class I and II - the two largest categories of

cytokine receptors - begins with ligand-induced dimerization of the receptor subunits. See also Baumann et al., Journal of Biological Chemistry, "Signaling by the Cytoplasmic Domain of Hematopoietic Receptors that Involves Two Distinguishable Mechanisms in Hepatic Cells", 269:23, 16297-16394 (1994) at p. 16297, col. 1 (copy enclosed as Exhibit 1), which expressly states that members of the hematopoietin receptor family (i.e., class I cytokine receptors) are structurally related and control proliferation or differentiation or both of many cell types through ligand induced dimerization of receptor subunits.[1]

Receptor clustering is also a key initiating step for the function of TNF family of cytokines.[2]

Thus, dimerization is the rule rather than the exception. Accordingly, given the well-characterized nature of cytokine receptors, applicants submit that cytokine receptors are not so "unpredictable" to give rise to the higher level of scrutiny suggested by the Office.

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[1] See also:
Heldin *et al.* Cell, "Dimerization of cell surface receptors in signal transduction", 80:213-223 (1995) - at p. 213, col. 1 : "Growth factors and cytokines exert their effects via binding to cell surface receptors...such receptors often are activated by ligand induced dimerization or oligomerization." (Copy enclosed as Exhibit 2)
Alexander *et al.*, EMBO, "Point mutation within a dimmer interface homology domain of c-Mpl induce constitutive receptor activity and tumorigenicity," 14:5569-5578 (1995) - at p. 5569, abstract: "[A] recurring mechanism for the activation of haematopoietin receptors is the formation of functional complexes by receptor subunit oligomerization." (Copy enclosed as Exhibit 3)

[2] See, for example:
Naismith JH, *et al.*, Structure, "Structures of the extracellular domain of the type I tumor necrosis factor receptor," 4(11):1251-62 (1996) - at p. 1251, abstract: "Signalling is thought to occur when a trimer of TNF binds to theextracellular domains of two or three receptor molecules, which permits aggregation and activation of the cytoplasmic domains." (Copy enclosed as Exhibit 4)
Menart V, et al. "Early events in TNFa-p55 receptor interactions--experiments with TNF dimers," Pflugers Arch. 439(3 Suppl):R113-5 (2000) - at p. 113, abstract: "The first essential step in TNF signal transduction is believed to be clustering of the membrane bound receptors around the trimeric TNF molecule." (Copy enclosed as Exhibit 5)

In sum, given the nature of the invention, the level of skill in the art, the representative nature of the disclosed embodiments, and the degree of guidance set forth in the specification as to the relevant, identifying characteristics defining members of the claimed genus, applicants have conclusively demonstrated that the subject matter of the claims would naturally occur to one of ordinary skill in the art upon reading the earlier specification such that he would recognize that applicants were in possession of the claimed invention.

For all of the aforementioned reasons, applicants' specification clearly conveys to the skilled person that applicants had possession of the claimed subject matter, the written description requirement of § 112 is therefore satisfied and the rejection should be withdrawn.

IV. Rejections Under 35 U.S.C. § 102

Claims 5, 6, 8, 10, 13, 15, and 17 stand rejected under 35 U.S.C. § 102(e) as being anticipated by Capon et al. (USPN 5,837,544). According to the Examiner,

> Capon teaches a chimeric construct comprising a ligand binding domain and a proliferation signaling domain (PSD), as well as vectors and cells containing said constructs. More particularly, the chimeric construct can comprise an inducer-responsive clustering domain (ICD), i.e., a hormone receptor domain, which upon binding the inducer or ligand dimerize or clusters. [citation omitted.] Furthermore, the ICD domains can be eukaryotic steroid receptor molecules, including estrogen, progesterone, and androgen. In addition, the PSD domains of the chimeric construct can be the transducing domains (i.e., proliferation domains) of cytokine receptors, such as IL-2 for example. [citation omitted.] Further, the PSD can be G-SCF. [citation omitted.]

For the following reasons, this rejection should be withdrawn.

As applied to amended independent claim 5, this rejection should be withdrawn. Claim 5 has now both been amended to recite that the second polypeptide includes an amino acid sequence in which a portion of the G-CSF extracellular domain has been deleted or a proliferation-inducing part thereof that, upon said dimerization of the first polypeptide, imparts proliferation activity to a cell. Support for this amendment can be found, for example, at page 9, lines 16-24, of the English language specification. Since Capon does not disclose such a polypeptide, Capon does not anticipate the present amended claim.

With respect to independent claim 8, applicants note Capon does not teach a vector comprising a gene encoding a fusion protein and a desired exogenous gene. Further, nowhere does Capon suggest designing such a vector having the claimed features. Thus, the Office's basis for the § 102 rejection is in error, and this basis of the rejection should be withdrawn.

V. **Provisional Obviousness-Type Double Patenting**

Claims 5, 6, 8, 10, 12, 14, 15, and 17-19 were provisionally rejected under the judicially created doctrine of obviousness-type double patenting as being unpatentable over claims 29-36, 39-47, and 49-50 of co-pending application no. 10/089932. Applicants acknowledge the Office's rejection and agree to address this rejection upon an

indication of otherwise allowable subject matter in this application.

CONCLUSION

Applicant submits that the claims are in condition for allowance, and such action is respectfully requested.

Enclosed with the concurrently filed Request for Continued Examination are a Petition to extend the period for replying to the final Office Action for three months, to and including February 24, 2005, and a check in the amount of the required fee.

If there are any additional charges or any credits, please apply them to Deposit Account No. 03-2095.

Respectfully submitted,

Date: 24 February 2005

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Exhibit 1

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 269, No. 23, Issue of June 10, pp. 16297-16304, 1994
Printed in U.S.A.

Signaling by the Cytoplasmic Domain of Hematopoietin Receptors Involves Two Distinguishable Mechanisms in Hepatic Cells*

(Received for publication, November 3, 1993, and in revised form, March 30, 1994)

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The receptor for granulocyte colony stimulating factor (G-CSFR) and chimeric receptors consisting of the extracellular domain of G-CSFR and the transmembrane and cytoplasmic domain of the leukemia inhibitory factor receptor, gp130, or c-*mpl* function as homodimeric complexes. These receptors mediate a similar stimulation of gene transcription via separate regulatory elements of acute phase plasma protein genes. To identify the receptor regions within the cytoplasmic domains necessary for transcriptional regulation, the receptors were transiently expressed in rat hepatoma cells. Each receptor form reconstituted G-CSF-induced expression of a chloramphenicol acetyltransferase gene construct containing the cytokine response element of the rat α_1-acid glycoprotein gene. This regulation required the presence of two conserved sequence motifs (referred to as box 1 and box 2) in the cytoplasmic domains of each receptor. With the exception of G-CSFR-MPL chimera, the receptors also mediated a similarly high stimulation via the IL-6 response element of the rat β-fibrinogen and hemopexin genes. Regulation of the IL-6 response element required, however, in addition to boxes 1 and 2, a third sequence motif (box 3). This motif is absent in the cytoplasmic domain of c-*mpl*, possibly explaining its inability to activate the IL-6 response element. When cells which express receptor forms with prominent box 3 function were treated with suramin, a ligand-independent gene stimulation via the IL-6 response element was observed. The suramin effect probably involves a receptor dimerization mediated by the extracellular G-CSFR domain and by the intracellular regions that include box 3.

Members of the hematopoietin receptor family are structurally related and control proliferation and/or differentiated cell functions of many cell types (1, 2). To achieve signaling, a ligand-induced dimerization or oligomerization of receptor subunits is required. In the example of the receptor for growth hormone (GH),[1] Epo, and G-CSF, homodimerization of the ligand-binding subunits is believed to be sufficient (3–5), whereas the receptors for IL-2, IL-3, IL-5, GM-CSF, and the gp130-dependent IL-6-type cytokine receptors depend upon formation of a heterodimer or oligomer of the ligand-occupied receptor (or α-) subunit with the signal transducing (or β-) subunit (6, 7).

The receptor subunits with signal-transducing capabilities show sequence similarities which extend to the cytoplasmic domains. To achieve a proliferative signal in hematopoietic cell lines, the receptor subunits require minimally the membrane proximal cytoplasmic region of approximately 65 amino acid residues which contain two conserved sequence motifs, termed box 1 and box 2 (5, 8–12). A similar requirement has been noted for the hematopoietin receptor-related oncogenic v-*mpl* encoded by the myeloproliferative leukemia retrovirus (13, 14).[2] Exceptions include the GHR, that produces a proliferative signal with just the box 1 motif (12) and the IL-4R, that mediates signaling independently of boxes 1 and 2 via a unique element located carboxyl-terminal to box 2 (15).

In a few examples, the receptor elements that confer regulation of differentiated gene expression and proliferation have been compared. The data available thus far indicate that these two types of cellular response are controlled by the same structure in GHR (16) and EpoR (17). In contrast, a third cytoplasmic receptor element (termed box 3) was necessary in addition to boxes 1 and 2 for stimulation of IL-6-sensitive APP genes in hepatic cells and of ciliary neurotrophic factor-sensitive neuropeptide gene in neuroblastoma cells by gp130, LIFR, and G-CSFR (18, 19) or for activation of myeloperoxidase and leukocyte elastase in myeloid cells by G-CSFR (20).

Since these structure/function analyses of the receptor domains have relied mainly on the use of different cell systems and assay techniques, direct comparison of the function of boxes 1, 2, and 3 among the redundantly acting hematopoietin receptors has not been possible. Moreover, based on the observation that, in hepatic cells, the receptors for IL-6-type cytokines similarly activated the expression of several APP genes, the action of a common singular signaling mechanism has been suggested (*e.g.* Refs. 21 and 22). When characterizing the regulation of several IL-6-responsive rat APP genes, we have observed that two separate types of cytokine response elements exist. In this paper, we describe that activation of gene transcription through these two response elements does not depend on identical receptor cytoplasmic domains. The data suggest that at least two distinguishable signaling mechanisms are operating in hepatic cells. The experimental system permits the functional characterization of the signal initiation by boxes 1, 2, and 3 of the IL-6-type cytokine receptor subunits, c-*mpl*, and probably other members of the hematopoietin receptor family.

MATERIALS AND METHODS

Cells—The subcloned line (T-7–18–1) recently derived from H-35 cell clone T-7–18 (23) was used because of its increased uptake of exogenous

* This study was supported by National Cancer Institute Grant CA26122 and a grant from the Buffalo Medical Foundation (to H. B.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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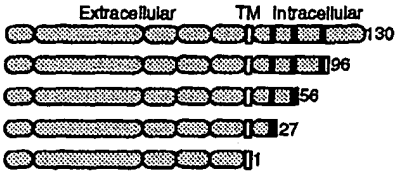
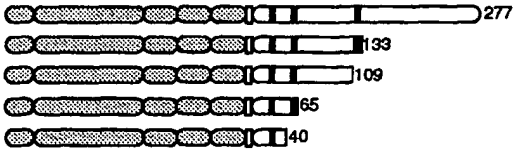
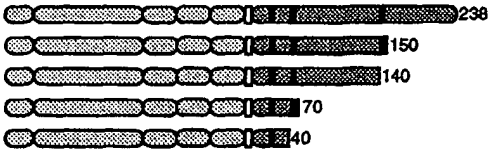
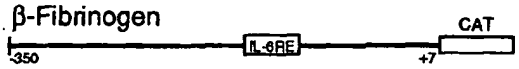
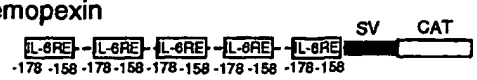
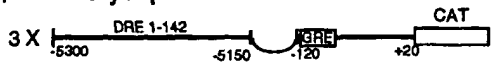
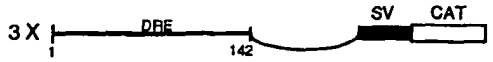
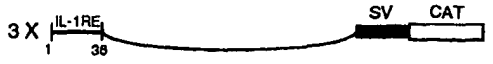
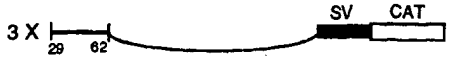
[1] The abbreviations used are: GH, growth hormone; AGP, α_1-acid glycoprotein; APP, acute phase plasma protein; CAT, chloramphenicol acetyltransferase; CytRE, cytokine response element; DRE, distal regulatory element; Epo, erythropoietin; βFB, β-fibrinogen; G-CSF, granulocyte colony stimulating factor; GM-CSF, granulocyte macrophage colony stimulating factor; GRE, glucocorticoid response element; LIF, leukemia inhibitory factor; MUP, major urinary protein; R, receptor.

[2] L. Benit, G. Courtois, M. Charon, P. Varlet, I. Dusanter-Fourt, and S. Gisselbrecht, submitted for publication.

TABLE I

List and schematic diagram of the relevant receptor expression vectors and reporter gene constructs used in this study

Plasmid	Name
Receptor Expression Vectors	
G-CSFR	
	pG-CSFR(130) pG-CSFR(96) pG-CSFR(56) pG-CSFR(27) pG-CSFR(Δcyto)
G-CSFR-gp130	
	pG-CSFR-gp130(277) pG-CSFR-gp130(133) pG-CSFR-gp130(109) pG-CSFR-gp130(65) pG-CSFR-gp130(40)
G-CSFR-LIFR	
	pG-CSFR-LIFR(238) pG-CSFR-LIFR(150) pG-CSFR-LIFR(140) pG-CSFR-LIFR(70) pG-CSFR-LIFR(40)
G-CSFR-MPL	
	pG-CSFR-MPL
gp130	
	pGp130
CAT Reporter Gene Constructs	
β-Fibrinogen	
	pβFB(350)-CAT
Hemopexin	
	prHX(5xIL-6RE)-CAT
α_1-Acid Glycoprotein	
	pAGP(3xDRE)-GRE-SV-CAT
	pAGP(3xGRE)-CAT
	pAGP(3xDRE 1-142)-SV-CAT
	pAGP(4xDRE 1-62)-SV-CAT
	pAGP(3xDRE 1-36)-SV-CAT
	pAGP(3xDRE 29-62)-SV-CAT
	pAGP(2xDRE 63-117)-SV-CAT

DNA. The cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal calf serum and antibiotics.

Plasmid DNAs—Table I lists the critical plasmids used in this study. The expression vector for the following receptor forms in pDC302 have been described before (numbers refer to the length of the cytoplasmic domains starting with the first amino acid residue following the transmembrane domains as 1): human G-CSFR(130(full-length of isoform D7)) G-CSFR(96), G-CSFR(56), G-CSFR(27), and G-CSFR(Δcyto; or 1) (18, 24), full-length human gp130 (25), IL-4R (26), and IL-7R (27). The chimeric receptors consisted of the extracellular domain of G-CSFR combined with transmembrane and cytoplasmic domain of human LIFR, human gp130, or murine c-*mpl* and include the following forms: G-CSFR-LIFR(238(full length), 133, 109, 65, and 40) (19), G-CSFR-gp130(277(full length) 131, 109, 65, 40) (19), and G-CSFR-MPL(121(full length)) (14). The murine full-length EpoR in expression vector pXM (28) was provided by Dr. J. N. Ihle, St. Jude Children's Research Hospital, Memphis, TN.

The reporter CAT gene construct included the previously described IL-6-responsive pratβFB(350)-CAT (29) and pratHx(5 × IL-6RE)-CAT (30) (provided by Drs. U. Mueller-Eberhard and S. Immenschuh, Cornell University, New York). As the multi-cytokine-responsive construct served pratAGP(3 × DRE)-GRE-CAT (originally called pAGP(3 × DRE)140-CAT) (31) and pAGP(3 × GRE)-CAT (29). Response-specific constructs consisted of pSV-CAT containing the 142-base pair DRE and subregions: 3 × DRE(1–142), 4 × DRE(1–62) (=CytRE); 3 × DRE(1–36) (=IL-1RE); 3 × DRE(29–62); and 2 × DRE(63–117) (=IL-6RE) (32). The internal transfection marker in all experiments was pIE-MUP (29).

Cell Transfection and Analysis—Optimal transfection of H-35 cells were obtained by the DEAE-dextran procedure (33). Briefly, the cells were plated at 1:4 dilution into 10-cm dishes. After 24 h, the DNA-DEAE-dextran complex in 1.5 ml of Tris-buffered saline was prepared and consisted of the following plasmid DNAs: 15 μg of CAT reporter gene construct, 3 μg of pIE-MUP, and 2.5 μg of receptor expression vector. The cells were incubated with this mixture for 40 min at 37 °C, then 4.5 ml of culture medium and 0.1 mM chloroquine were added, and incubation continued for 3 h. The cells were shocked for 3 min at room temperature with 20% glycerol in Tris-buffered saline (approximately 30% of the cells were killed). After overnight recovery, the transfected cell cultures were divided into 6-well cluster plates. After 24 h, the subcultures were treated for an additional 24 h with serum-free minimal essential medium containing either 1 μM dexamethasone alone (= control) or in addition 100 ng/ml human recombinant IL-4, IL-6 (Genetics Institute), IL-7, or G-CSFR, 40 units/ml Epo (Amgen), 10 ng/ml IL-1β, or 0.5 mM suramin (provided by Dr. G. Strassmann, Otsuka America Pharmaceutical). The cells were extracted in 100 μl of 0.5 M Tris, pH 7.8. After a 5-min incubation at 65 °C, 1 to 15 μl of extract was used to determine CAT activity. The final culture medium was subjected to immunoelectrophoresis to quantitate expression of the internal marker MUP (29). The CAT activity for each culture was normalized to the amount of MUP and then calculated relative to the control culture in each experimental series.

Receptor RNA Analysis—The amount of expression vector for G-CSFR and chimeric receptors (2.5 μg/1.5 ml) ensured maximal receptor response in all H-35 cell transfection experiments. Higher amounts of expression vector (tested 5 and 10 μg/1.5 ml) did not enhance the receptor signal. As described previously (19), the relative low efficiency of DNA uptake by H-35 cells precluded a quantitation of receptor proteins expressed in the transiently transfected cell cultures by either radioligand binding or Western blotting. We have, however, verified by transient transfection into CV1/EBNA cells that every receptor construct used in this study (with the exception of EpoR that had not been tested) directed the synthesis and surface display of similar levels of receptor binding sites and that these showed the appropriate ligand binding activities (19, 26, 27).[3] It was reasonable to assume that the expression of the same receptor vectors in transiently transfected H-35 cells was qualitatively similar to CV1/EBNA cells, albeit at a much lower level. The similar expression of receptor vectors in H-35 cells could be determined by Northern blot analysis. H-35 cells in 15-cm culture dishes were transfected with 3 ml of DEAE-dextran solution containing 30 μg of expression vector for G-CSFR or G-CSFR chimeras. Following a 36-h recovery period, RNAs were extracted (34), and the polyadenylated fractions were enriched by chromatography on oligodeoxythymidine-cellulose spin columns. Twenty μg of polyadenylated RNA were separated on formaldehyde-containing agarose gel. After transfer of the RNA to Nytran® membrane (Schleicher and Schuell), the section with RNA larger than 18 S rRNA was hybridized to a ^{32}P-labeled 1 kilobase cDNA fragment encoding the extracellular domain of human G-CSFR. The section with RNA smaller than 18 S rRNA was hybridized to full-length cDNA for the housekeeping enzyme, triosephosphate isomerase (35) that served as internal marker for RNA loading (36). The expression of mRNA for progressively truncated G-CSFR and G-CSFR-gp130 is illustrated in the representative experimental series shown in Fig. 1.

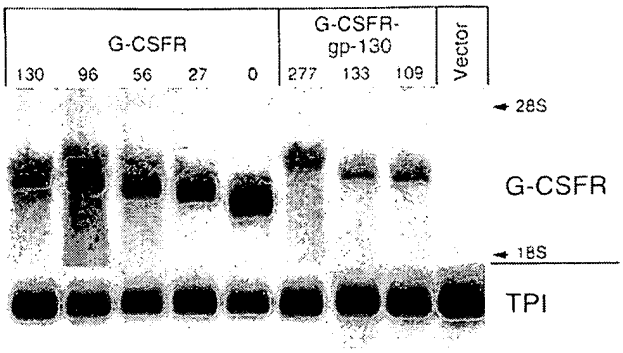


FIG. 1. **Expression of the mRNA for full-length and truncated G-CSFR and G-CSFR-gp130 in transiently transfected H-35 cells.** Polyadenylated RNA (20 μg per lane) from H-35 cells transiently transfected with the receptor forms indicated at the *top* were analyzed by Northern blot hybridization as described under "Materials and Methods." The autoradiogram of the *upper section* with receptor mRNA (*G-CSFR*) was exposed for 12 days, the *lower section* with triosephosphate isomerase (*TPI*) mRNA for 16 h. The positions of the 28 S and 18 S rRNA are indicated.

RESULTS

IL-6-type Cytokine Receptors Generate Two Separate Signals for Activating APP Genes—The receptors for IL-6 and LIF appear to exert a qualitatively similar, if not identical, signaling event in hepatoma cells resulting in the transcriptional activation of the IL-6-responsive APP genes. The only distinguishing feature among the receptors is the magnitude by which the target genes are activated (19). In order to define the structural elements within the cytoplasmic domains of the signaling receptor subunits that elicit the presumed common action, we needed to study the cytoplasmic domains without potential interference by endogenous receptor subunits. To do so, these domains were incorporated into chimeric receptors that utilized the extracellular domain of G-CSFR (18, 19, 25). These chimeric receptors, like the *bona fide* G-CSFR, reconstituted a G-CSF-dependent activation of the co-transfected IL-6-responsive βFB-CAT gene construct in rat hepatoma H-35 cells (Fig. 2). The magnitude of stimulation varied among the receptor forms, with the most prominent action achieved with G-CSFR-gp130, followed with G-CSFR and then with G-CSFR-LIFR (Fig. 2*B*).

By progressive carboxyl-terminal deletion, we have previously defined the minimal size cytoplasmic domain of G-CSFR, gp130, and LIFR required for βFB-CAT gene stimulation (19). Each receptor contained within this domain a conserved sequence motif referred to as box 3 which, when removed, essentially eliminated all signal activity (Fig. 2, *A* and *B*). The failure to detect signaling by box 3-deficient receptors was not attributed to lack of expression of these receptor forms in transfected cells (Fig. 1, Ref. 19, note under "Materials and Methods"). In fact, the signaling function of these truncated receptors could be demonstrated by using an alternative reporter construct that contained the multi-cytokine- and hormone-responsive regions of the rat AGP gene. When the same receptor forms were tested for their ability to stimulate pAGP(3XDRE)-GRE-CAT, the removal of the box 3 element did not correlate with loss of G-CSF responsiveness (Fig. 2*A*). Although the three receptor forms showed differences in the relative activities to regulate the two reporter gene constructs, cross-comparison was facili-

[3] H. Baumann, D. Gearing, and S. F. Ziegler, unpublished data.

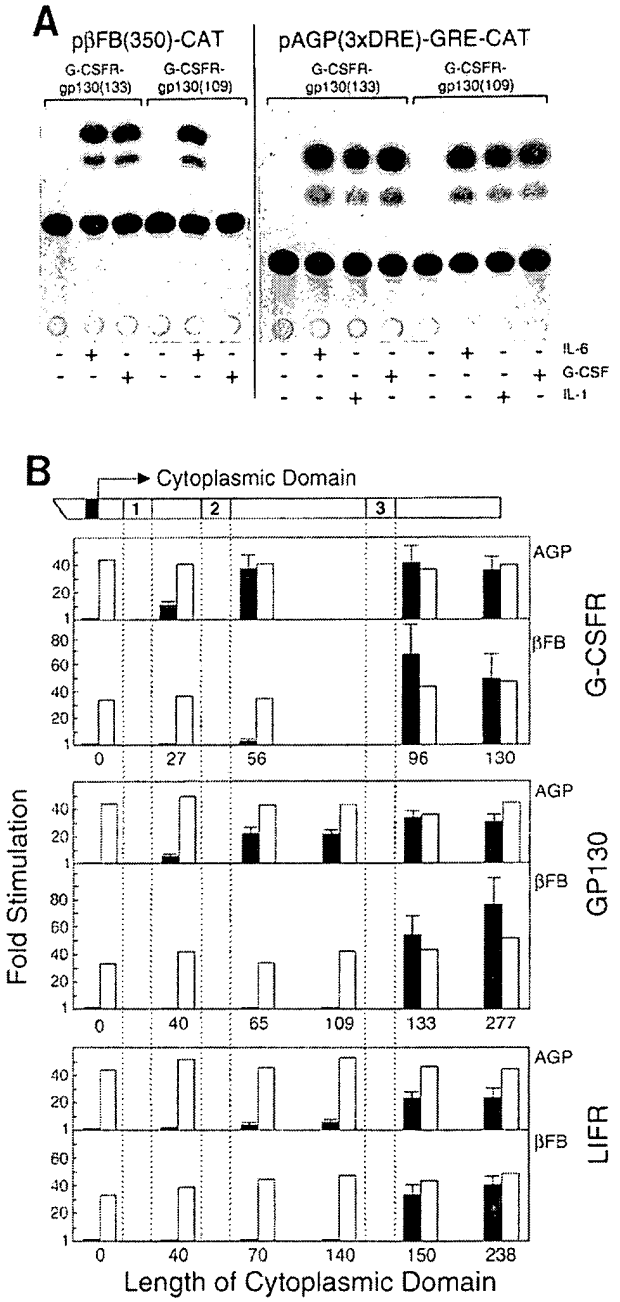


FIG. 2. **Receptor-specific regulation of APP gene elements.** H-35 cells were transfected with expression vectors for G-CSFR or the chimeric G-CSFR-gp130 or G-CSFR-LIFR containing the indicated length of cytoplasmic domains. The reporter gene constructs were either pβFB(350)-CAT (*βFB*), or pAGP(3XDRE)-GRE-CAT (*AGP*). The cells were treated with IL-6 (*open bar*), G-CSF (*closed bar*), and IL-1 as indicated. The CAT activity in each subculture was determined (*A*) and normalized to the control culture in each experimental series (*B*). In *A*, the results of one representative experiment is shown documenting the box 3-dependent signaling effect in G-CSFR-gp130. In *B*, the quantitative values for the relative receptor activities were determined in several separate experimental series. Each construct was tested at least in two independent experiments (shown mean values). For those constructs which had been included in three to six independent experiments, mean ± S.D. is shown. The position of the box 1, 2, and 3 motifs in each receptor form is indicated at the *top*.

tated by using the endogenous IL-6 response as a reference point (Fig. 2*B*).

The maximal regulation of the AGP-CAT construct by G-CSFR required just the box 1 and box 2 region (G-CSFR(56)). Inclusion of box 3 (G-CSFR(96)) did not significantly modify the response, and, interestingly, G-CSFR(27), containing only the box 1 motif, showed appreciable activity.

The G-CSFR-gp130 constructs required all three boxes for maximal activity. Removal of box 3 (G-CSFR-gp130(109)) reduced the activity by as much as 2-fold (Fig. 2*A*). Deletion of boxes 2 and 3 resulted in a low, yet detectably active, receptor (G-CSFR-gp130 (40)) (Fig. 2*B*).

The activity of G-CSFR-LIFR differed from that of the two other receptor forms by lower stimulation of both βFB- and AGP-CAT constructs, and the activity was more strongly dependent on the box 3 motif. Removal of box 3 (G-CSFR-LIFR(140)) resulted in a drastically reduced stimulation of the AGP-CAT construct. With the additional deletion of box 2 (G-CSFR-LIFR(40)), the remaining receptor protein was inactive (Fig. 2*B*).

We concluded from these results that the three receptor forms did not deliver one but at least two types of signals, one derived from boxes 1 and 2 and targeted to the cytokine RE of AGP gene (termed "cytokine" signal), and the other derived from boxes 1 through 3 and targeted to both the cytokine RE of AGP gene and to the IL-6RE of βFB gene (termed "IL-6" signal). There were characteristic differences among the receptors: G-CSFR produced both a strong IL-6 and a cytokine signal, whereas G-CSFR-gp130 produced a strong IL-6 signal but a much reduced cytokine signal, and G-CSFR-LIFR produced essentially only an IL-6 signal.

MPL Delivers a Cytokine Signal in H-35 Cells—Since the cytoplasmic domains of several other hematopoietin receptor subunits contain the functionally relevant box 1 and box 2 motifs, the possibility existed that such receptors produced a cytokine signal in hepatic cells similar to the IL-6-type cytokine receptors. Therefore, we transfected expression vectors for IL-4R, IL-7R, EpoR, and G-CSFR-MPL, together with either AGP- or βFB-CAT reporter gene construct into H-35 cells. No signaling event was detectable in the cells receiving IL-4R, IL-7R, or EpoR (data not shown). By contrast, G-CSFR-MPL reconstituted a prominent G-CSF response of the AGP-CAT gene but not of the βFB-CAT gene (Fig. 3*A*).

There are several possible explanations for not observing an IL-6 signal delivered by these receptors: (*a*) the receptors do not have a box 3 motif in the cytoplasmic domains; (*b*) the receptors require additional signaling subunits not present in H-35 cells; or (*c*) the regulation via the IL-6RE of the βFB gene is restricted to IL-6-type cytokine receptor subunits. Therefore, to assess the third possibility, we included in our functional analyses, CAT reporter gene construct containing the IL-6-responsive elements of other rat APP genes (37). No signaling was detected with the IL-6 response elements from the thiostatin and haptoglobin genes (data not shown) or hemopexin gene (Fig. 3*B*). In separate transfection experiments, we verified that all of these gene elements were appropriately activated by the box 3-containing G-CSFR (Fig. 3*B*), G-CSFR-gp130, and G-CSFR-LIFR (data not shown).

From the fact that G-CSFR-MPL, in contrast to IL-4R, IL-7R, or EpoR, was able to regulate via the cytokine RE of the AGP gene, we concluded that G-CSFR-MPL probably did not need additional receptor subunits for signaling and, when homodimerized, could trigger a cytokine signal. Since the G-CSFR-MPL was devoid of a detectable IL-6 signal (Fig. 3), this receptor construct was well suited to define more precisely the genetic target of the cytokine signal.

Identification of the Regulatory Element Responding to the Cytokine Signal—Considering that the AGP(3 × DRE)-GRE-CAT construct initially used to assess receptor signaling was a composite of multiple response elements (31, 32), the target of the cytokine signal generated by G-CSFR-MPL and the IL-6-

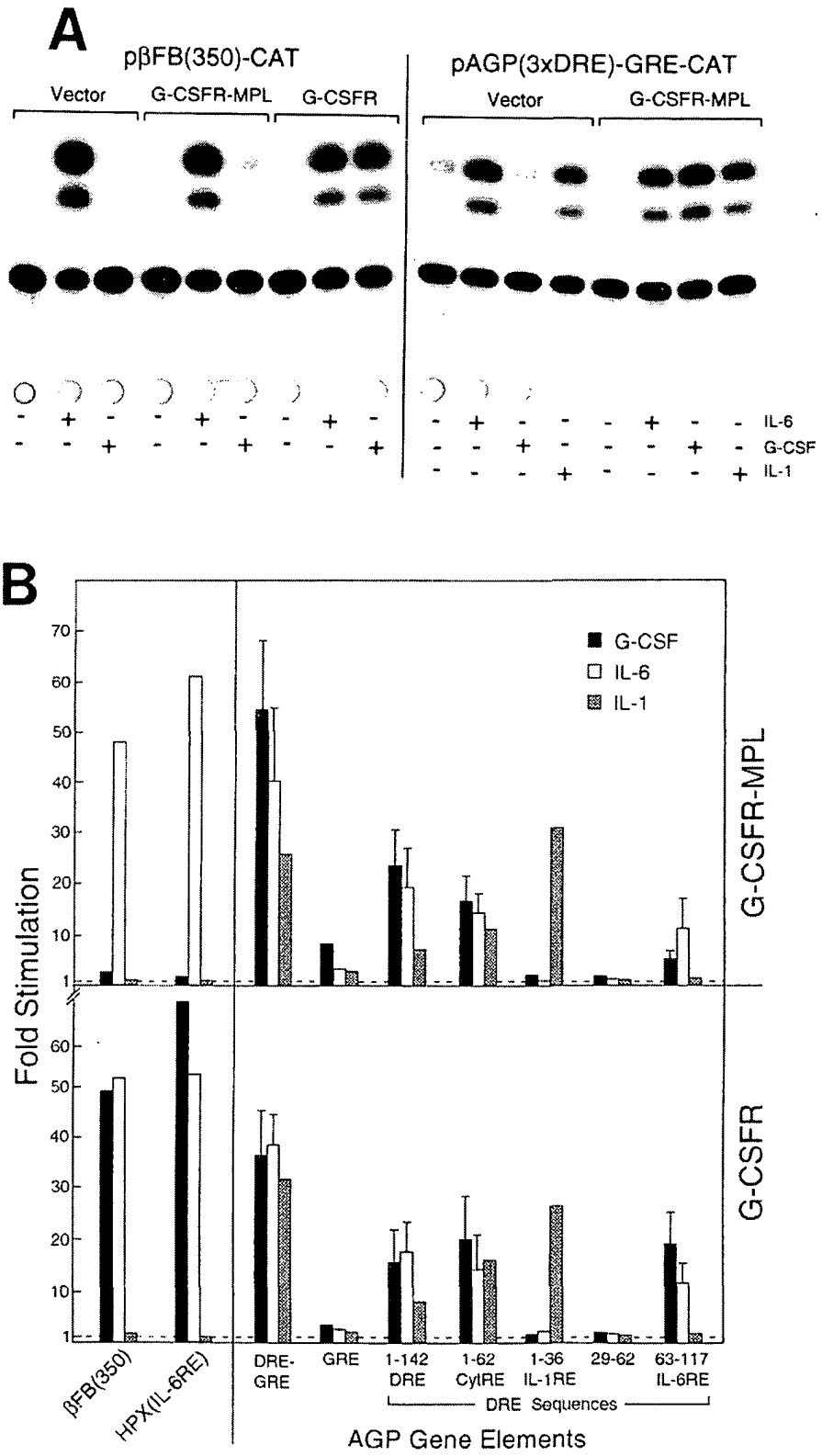


FIG. 3. **Activity of G-CSFR-MPL in H-35 cells.** H-35 cells were transfected with a combination of expression vector for G-CSFR (full length) or G-CSFR-MPL and reporter CAT gene construct containing the indicated regulatory elements. In *A*, the result of one experiment is reproduced illustrating the regulatory element specific action of G-CSFR-MPL. In *B*, the relative activities of the receptor forms in regulating specific response elements are compared (means of two, or means ± S.D. of three independent experiments).

type cytokine receptors was not evident. To assess the relative contribution of specific AGP gene sequences, CAT reporter constructs carrying separate AGP gene elements (Table I) were assayed (Fig. 3*B*). The GRE and proximal promoter region showed a minor response. The 142-base pair DRE region served as the principle target for cytokine signal. A major contribution to the observed response was mediated by the region from position 1 to 62 (termed CytRE). The subfragment from position 1–36 (representing the specific IL-1RE (32)) failed to respond to the G-CSF signal as did the complementary sequence tested in the context of the region 29–62. The DRE-internal IL-6RE at position 63 to 117 (32) was, like other IL-6REs,

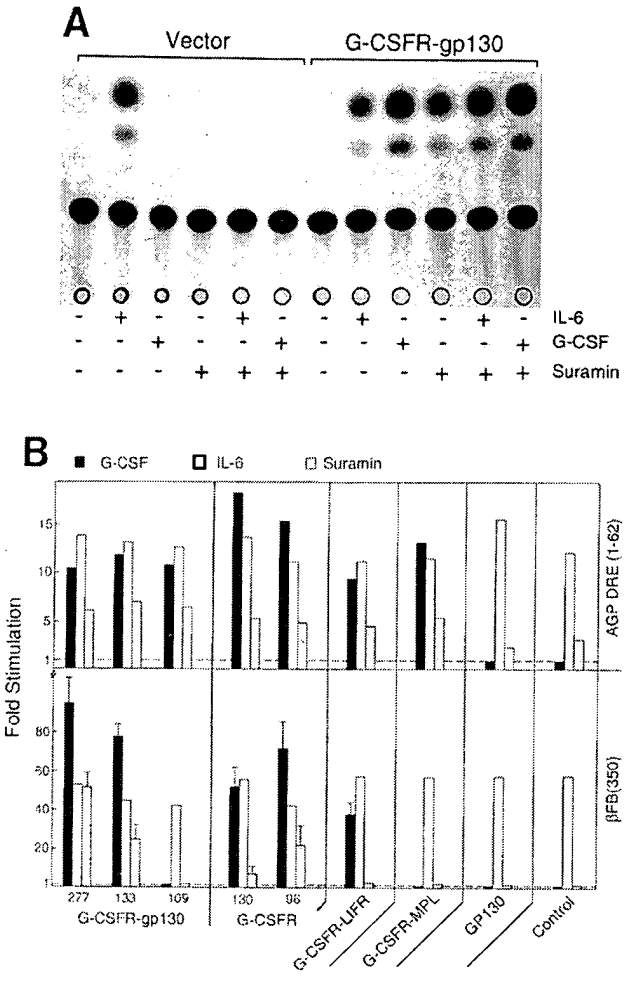


FIG. 4. **Effect of suramin on receptor function.** H-35 cells were transfected with the indicated receptors together with either the pβFB(350)-CAT (*A* and *B*) or pAGP(4 × DRE(1–62)-SV-CAT (*AGP DRE (1–62)* in *B*). The cells were treated with IL-6, G-CSF, or suramin. In *A*, the characteristic suramin effects on IL-6 regulation in control cells and the G-CSFR-gp130(277) transfected cells are illustrated. In *B*, the relative activities of the receptor constructs are compared (means of two and means ± S.D. of three separate experiments).

preferentially responsive to G-CSFR and much less to G-CSFR-MPL. Taken together, the data suggest that the cytokine signal generated by G-CSFR and MPL was primarily targeted to the CytRE, and the regulating event involved a cooperativity with the *trans*-acting elements associated with the IL-1RE.

Suramin Acts As an Activating Agent of the IL-6 Signal by G-CSFR and G-CSFR Chimeras—Recently we have noted that suramin, a polysulfated naphthyl urea, inhibited the agonist activation of IL-6-type cytokine receptors in H-35 cells. This was attributed in the case of IL-6 to prevention of the ligand interaction with the cell surface receptor subunits (38). In an attempt to verify that this fairly nonspecific inhibitory effect also applied to the signaling mediated by G-CSFR and G-CSFR chimeras, we extended the same analysis to G-CSFR-transfected cells. While suramin at 0.5 mM was capable of preventing IL-6 stimulation of the βFB-CAT construct in control cells, it did not affect the response to G-CSF (Fig. 4*A*). More surprising was that suramin alone acted as a G-CSF-like agonist in cells that have received G-CSFR and G-CSFR-gp130 chimera but not with cells with G-CSFR-LIFR. The agonistic properties of suramin correlated with the ability of these receptor forms to respond to G-CSF and to generate a high level stimulation via IL-6REs (Fig. 4*B*). This correlation was particularly impressive in the case of G-CSFR which, when truncated to a 96-residue cytoplasmic domain, gained an enhanced IL-6 signal (Figs. 2*B* and 4*B*) and concomitantly increased suramin response (Fig. 4*B*). The specificity of this suramin effect on the IL-6RE was demonstrated by the fact that the reporter gene expression via the CytRE was minimally affected.

The suramin stimulation was most prominent with G-CSFR-gp130(277), suggesting that the carboxyl-terminal gp130 sequences from residues 110 to 277, including box 3, were critical for suramin action. Suramin was ineffective in stimulating βFB-CAT expression in control cells (Fig. 4, *A* and *B*), suggesting that either the endogenous gp130, unlike the G-CSFR-gp130 chimera, was not activated by suramin, or the concentration of endogenous gp130 was too low to promote suramin-mediated complex formation. The latter possibility could be ruled out by the observation that overexpression of human gp130 did not promote a suramin response (Fig. 4*B*).

From these results we concluded that suramin may serve as a new experimental reagent for specifically inducing box 3-dependent signaling events by G-CSFR in the absence of the *bona fide* receptor ligand.

DISCUSSION

In this study, we demonstrate that hematopoietin receptors have the potential to elicit two types of signaling events in hepatic cells. One type, the cytokine signal, is dependent upon the cytoplasmic domain motifs of box 1 and box 2 and is targeted to the CytRE of the rat AGP gene. The second type, the IL-6 signal, is only manifested if the combination of boxes 1–3 are present, can be artificially induced by suramin, and has as specific genetic target the IL-6RE that contains the consensus sequence C(T/C)GGGAA (32).

The receptor requirement for gaining the cytokine signal that regulates the AGP gene elements in H-35 cells appears to coincide with the requirement for a proliferative signal in stably transfected, factor-dependent BAF pre-B cell line. BAF cells transfected with cytoplasmic deletion mutants of the G-CSFR and gp130 demonstrated that only box 1 and box 2 were required for a proliferative signal (8, 18), as for stimulating CAT gene expression via the CytRE (Fig. 2*B*). In contrast, the chimeric G-CSFR-LIFR(238) construct failed to deliver a proliferative signal in BAF cells (39) while it functioned to activate the IL-6RE in hepatoma cells (25). The fact that G-CSFR-LIFR(140) was unable to maintain a strong activation of the CytRE in H-35 cells (Fig. 1*B*) suggested that this truncated LIFR receptor cytoplasmic domain, when assayed in isolation, is deficient in the box 1 and 2 signal. One explanation for this is the presence of a tyrosine residue (Y11) in the LIFR box 1 where both the G-CSFR and gp130 encode tryptophan. Murakami *et al.* (8) have shown that changing this tryptophan to tyrosine in gp130 results in loss of the proliferative signal in BAF cells. Our current working model is that the same signal originating from box 1 and 2 of the G-CSFR, gp130, and c-*mpl* is responsible for proliferation in BAF cells and activation of the CytRE in H-35 cells, and that LIFR is incapable of generating this signal. The ability of measuring the signal mediated by box 1 and 2 motif in transiently transfected cells at the level of gene regulation will greatly facilitate its biochemical and molecular analysis and will permit, for the first time, a quantitative comparison with the box 3-dependent signaling pathway.

Potential candidates for communicating the receptor activation to the intracellular signaling pathways can be proposed for the protein tyrosine kinases JAK1, JAK2, and Tyk2 which have been found to be recruited by ligand activated cytokine receptors containing the box 1 and 2 motifs (16, 22, 28, 40, 41). The

downstream cytokine signal communication to the nucleus appears to converge in part with signals derived from growth factor receptors by utilizing common transcription factors such as p91 (42) or "stat" proteins (43) whose cytokine-dependent phosphorylation promotes translocation from cytoplasm to nucleus and integration into DNA binding complexes. At present, we do not know which kinases are activated by the transfected receptor constructs nor do we have any evidence for the involvement of specific stat proteins (*e.g.* p91 or related proteins) for regulating the reporter genes. The CytRE of the AGP gene does not contain a consensus sequence related to interferon-sensitive or *cis*-inducible elements (32, 43), is not activated by treatment of the cells with either interferon-α, -β, or -γ, or epidermal growth factor (data not shown), and does not serve as a target for a cytokine-inducible nuclear protein (44, 45).[4] Hence, the *trans*-acting factor(s) functioning in cooperation with C/EBP isoforms (37, 44) on the CytRE has yet to be defined.

The presence of box 3 motif determines whether the receptor is capable of generating an IL-6 signal in hepatic cells. The IL-6 signal-communicating factor to the IL-6RE is still ill defined. Transfection experiments and *in vitro* protein/DNA interaction studies have ruled out a critical role of isoforms for C/EBP, AP-1, and NF-κB (37, 45, 46) and indicated a functional distinction between IL-6RE and the CytRE (this paper). Gel mobility shift data indicated an IL-6-inducible nuclear binding protein present in liver and hepatoma cells as well as in cytokine-treated, nonhepatic cells. One binding activity, termed APRF, has been observed to be rapidly induced by IL-6 in the cytoplasm followed by nuclear translocation (22, 46) and appears to be identical with the *cis*-inducible factors SIF-A, -B, and -C (43). A second activity, termed IL-6REBP, was recorded to appear with delayed kinetics coinciding with the transcriptional activation of the marker APP gene, α_2-macroglobulin (45). The functional relevance of either factor in our system remains to be established.[4]

Suramin is considered to bind nonspecifically to proteins and, by doing so, preventing, for instance, the binding of peptide hormones to their receptors or inhibiting receptor subunit complex formation (38, 47). In the case of G-CSFR and chimeras, an inverse action seems to occur, suramin mediates a ligand-independent activation. A conceivable and yet to be documented mechanism would involve a suramin-mediated dimerization of receptor subunits. A receptor-dimerizing activity via the sulfate side groups of suramin could be envisioned that is mechanistically related to the artificial activation of signaling achieved by synthetic ligands, such as FK1012, in Jurkat cells (48). The results from the functional analyses of the various functional receptor forms suggested that both the extracellular domain of G-CSFR and the carboxyl-terminal part of the intracellular receptor domain, including the box 3 motif, participate in the suramin reaction. The involvement of the extracellular G-CSFR domain has been inferred from the observation that overexpression of G-CSFR(Δcyto), that exerts a strong, dominant negative action on G-CSFR function, similarly suppressed suramin activation (data not presented). The suramin-mediated receptor change seems to favor activation of the IL-6 signal with a relatively minor effect on the cytokine signal (Fig. 4*B*). Suramin action appears to predominate at the receptor level. We have no evidence of a modulation of receptor-downstream signaling events by suramin. For instance, the activation of the CytRE regulation by G-CSF through G-CSFR(56), G-CSFR-gp130(109), or G-CSFR-MPL in the presence of suramin is not affected (data not shown). Although in this system suramin acts in a nonphysiological manner, it nevertheless emerges as a useful reagent to assess whether a new hematopoietin receptor structure is capable of generating an IL-6 signal in hepatic cells. Moreover, recognizing the potential of suramin to activate signaling through G-CSFR adds a new aspect that has to be considered when applying this drug *in vivo*. One would predict that all those cells carrying G-CSFR will be stimulated by suramin treatment.

This study illustrates a new feature of hematopoietin receptors. These receptors not only share structural similarities, but also exert compatible signaling functions. When introduced into hepatoma cells, the receptors mediate signaling processes that can be defined by the transcriptional regulation via specific APP gene elements. The response of the hepatoma cells indicated the involvement of at least two signaling mechanisms that are controlled by subregions of the cytoplasmic receptor domain. The relative activities of these subregions probably contribute to the receptor-specific effects on proliferation and differentiated cell function.

Acknowledgments—We thank Dr. James N. Ihle for providing murine EpoR expression vector, Drs. Ursula Muller-Eberhard and Stephan Immenschuh for pratHx(5 × IL-6RE)CAT, Dr. Gideon Strassmann for suramin, Genetics Institute for human recombinant IL-6, Karen K. Morella and Yanping Wang for technical assistance, and Marcia Held for secretarial help.

[4] C.-F. Lai, S. Immenschuh, D. P. Gearing, S. F. Ziegler, and H. Baumann, submitted for publication.

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Cell, Vol. 80, 213–223, January 27, 1995, Copyright © 1995 by Cell Press

Exhibit 2

Dimerization of Cell Surface Receptors in Signal Transduction

Review

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Introduction

Cell growth, differentiation, migration, and apoptosis are in part regulated by polypeptide growth factors or cytokines. As these factors are unable to pass the hydrophobic cell membrane, a fundamental question is how they transduce their signals into the cell. Growth factors and cytokines exert their effects via binding to cell surface receptors; results obtained during recent years have given ample evidence that such receptors often are activated by ligand-induced dimerization or oligomerization. Moreover, the elucidation of intracellular signal transduction pathways have revealed that the activity of several components in these pathways are also regulated by dimerization. For instance, certain of the cytoplasmic signal transduction molecules dimerize after activation, and the active form of transcription factors are often dimers. It thus appears that dimerization is a mechanism of general applicability for the regulation of signal transduction.

This review focuses on the role of dimerization of cell surface receptors in signal transduction. Dimerization or oligomerization have been shown to occur after binding of several polypeptide hormones, cytokines, growth factors, or growth inhibitors to their receptors. Examples include protein-tyrosine kinase receptors, cytokine receptors, antigen receptors, receptors for tumor necrosis factor (TNF) and related factors, and serine/threonine kinase receptors (Figure 1; Table 1). There are, however, many variations on the theme, as will be discussed below.

Protein-Tyrosine Kinase Receptors

Many traditional growth factors, such as platelet-derived growth factor (PDGF), epidermal growth factor (EGF), and fibroblast growth factor (FGF), bind to receptors with tyrosine kinase activity (Table 1). Protein-tyrosine kinase receptors consist of single transmembrane domains separating the intracellular kinase domains from extracellular domains, which typically contain one or several copies of immunoglobulin-like domains, fibronectin type III–like domains, EGF-like domains, cysteine-rich domains, or other domains (reviewed by Fantl et al., 1993). Based on their structural characteristics, the tyrosine kinase receptors can be classified into families; the largest families are listed in Table 1.

Several of the ligands for protein-tyrosine kinase receptors are dimeric molecules, which thus contain two identical receptor-binding epitopes. Examples include PDGF and colony-stimulating factor 1 (CSF-1), which are disulfide-bonded dimers, and stem cell factor (SCF), which is a dimer held together by noncovalent forces. These ligands form stable receptor dimers by simultaneously binding two receptors. In addition to the bridging of the ligand between two receptors, it is possible that direct interactions between the receptors, involving epitopes located outside the ligand-binding domains, are important for stabilization of the receptor dimer. In the case of the SCF receptor, evidence has been presented that epitopes in the fourth immunoglobulin domain are involved in such receptor–receptor interactions (Blechman et al., 1995). It is possible that such direct receptor–receptor interactions are promoted by conformational changes in the receptors induced by ligand binding. Other ligands, like EGF, have apparent monomeric configurations; interestingly, however, recent calorimetric studies have shown that a single EGF molecule also can bind simultaneously to two receptor molecules (Lemmon and Schlessinger, 1994). Another variation on the theme is exemplified by ligands for Eph-related tyrosine kinase receptors. These ligands are cell surface attached and do not activate receptors in soluble form. The possibility that receptor dimerization or clustering is involved in receptor activation, presumably facilatated by membrane attachment of ligands, is supported by the finding that antibody-mediated clustering of soluble receptors led to activation of receptors (Davis et al., 1994).

Receptor Autophosphorylation

Dimerization of protein-tyrosine kinase receptors is followed by receptor "autophosphorylation," which mainly occurs by one receptor molecule phosphorylating the other in the dimer (Ullrich and Schlessinger, 1990). The autophosphorylation occurs on two principally different classes of tyrosine residues. On one hand, autophosphorylation is commonly seen on a conserved tyrosine residue within the kinase domains (Tyr-857 in the PDGF β receptor; Figure 2). In the cases of the receptors for insulin and hepatocyte growth factor (HGF), phosphorylation of the tyrosine residue at this and neighboring sites leads to an increase in the kinase activity and precedes phosphorylation of other sites in the receptor or substrates (Naldini et al., 1991; White et al., 1988). This thus appears to be an allosteric site that regulates the V_{max} of the receptor kinase. It is still not known how the autophosphorylation is initiated; one possibility is that the monomeric receptor has a low basal kinase activity, which is sufficient to phosphorylate and activate the companion receptor after dimerization. This would then rapidly be followed by reciprocal phosphorylation. Alternatively, the interaction between the intracellular domains of the receptors in the dimer may induce a conformational change that leads to an increased kinase activity. Not all receptors are regulated by phosphorylation inside the kinase domain, e.g., in the EGF receptor, the conserved tyrosine residue in the kinase domain appears not to be autophosphorylated.

The other class of autophosphorylation sites are normally localized outside the kinase domains and serve the important function of creating docking sites for downstream signal transduction molecules containing Src-homology 2 (SH2) domains. The SH2 domains consists of about 100 amino acid residues folded in such a way

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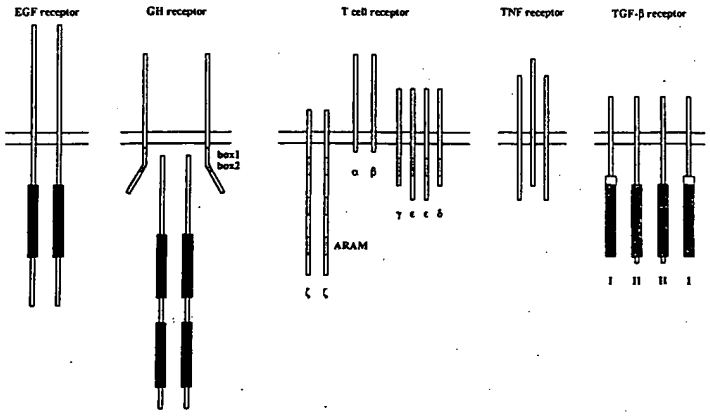

Figure 1. Examples of Receptors Activated by Dimerization or Oligomerization

Schematic representations of the complexes formed after ligand binding to receptors from the families discussed in the text, i.e., protein-tyrosine kinase receptors (the EGF receptor shown as an example), cytokine receptors (GH receptor bound to JAK kinases), antigen receptors (TCR), trimeric receptors (TNF receptor), and serine/threonine kinase receptors (TGFβ receptor). Tyrosine kinase domains are closed and serine/threonine kinase domains dark stippled. Box1, box2 (light stippled), refers to a region in cytokine receptors to which JAK kinases bind. ARAM (light stippled) refers to antigen recognition activation motifs in different subunits of antigen receptors that become phosphorylated by tyrosine kinases of the Src family and thereafter bind tyrosine kinases of the ZAP-70/Syk family.

Table 1. Families of Receptors Activated by Dimerization or Oligomerization

Receptor Type	Family	Examples	Characteristics
Protein-tyrosine kinase receptors	PDGF receptor family	PDGFR-α, PDGFR-β, SCFR (Kit), CSF-R (Fms), Flk-2	Five immunoglobulin-like domains extracellularly
	EGF receptor family	EGFR (ErbB), ErbB2 (Neu), ErbB3, ErbB4	Two cysteine-rich domains extracellularly
	FGF receptor family	FGFR-1, FGFR-2, FGFR-3, FGFR-4	Two to three immunoglobulin-like domains extracellularly
	IGF receptor family	insulin R, IGF-1R	Disulphide-bound heterotetramer of α and β chains
	HGF receptor family	HGFR (Met), MSPR (Ron)	Extracellular domain cleaved into an α and β chain
	VEGF receptor family	Flt-1, Flk-1 (KDR)	Seven immunoglobulin-like domains extracellularly
	Neurotrophin receptor family	Trk, TrkB, TrkC	
	Eph receptor family	Eph, Elk, Eck, Cck5, Sek, Eck, Erk	Two FNIII-like domains and a cysteine-rich domain extracellularly
Cytokine receptors	Class I cytokine receptor family		
	GH receptor subfamily	GHR, EPOR, PRLR, G-CSFR	Form homodimers
	IL-3 receptor subfamily	IL-3R, GM-CSFR, IL-5R	Form complexes with the β_c subunit
	IL-6 receptor subfamily	IL-6R, LIFR, CNTFR, IL-11R	Form complexes with gp130
	IL-2 receptor subfamily	IL-2Rα, IL-2rβ, IL-4R, IL-7R	Form complexes with IL-2Rγ
	Class II cytokine receptor family	IFN-α/βR, IFN-γRα, IFN-γRβ, IL-10R	
TNF receptor family		TNFR-1, TNFR-II, LNGFR, CD40, OX-40, Fas, CD27, CD30	Form trimers
Antigen receptors		TCR	Complex of α, β, γ, δ, ε, ζ an η subunits
		BCR	Complex of IgM and heterodimers of α/β subunits
Serine/threonine kinase receptor family	Type II receptor family	TGFβR-II, ActR-II, ActR-IIB	Form hetero-oligomers with type I receptors, i.e., TGFβR-I, ActR-1, ActR-1B, BMPR-IA, BMPR-IB, ALK-1

Receptor families and subfamilies discussed in the text are presented. Abbreviations used: R, receptor; PDGF, platelet-derived growth factor; SCF, stem cell factor; CSF, colony-stimulating factor; EGF, epidermal growth factor; FGF, fibroblast growth factor; IGF, insulin-like growth factor; HGF, hepatocyte growth factor; MSP, macrophage-stimulating protein; VEGF, vascular endothelial growth factor; FN, fibronectin; GH, growth hormone; EPO, erythropoietin; PRL, prolactin; IL, interleukin; LIF, leukemia inhibitory factor; CNTF, ciliary neurotrophic factor; IFN, interferon; TNF, tumor necrosis factor; LNGFR, low affinity nerve growth factor receptor; TCR, T cell receptor; BCR, B cell receptor; TGFβ, transforming growth factor β; Act, activin; BMP, bone morphogenic protein. Alternative designations are given within parentheses.

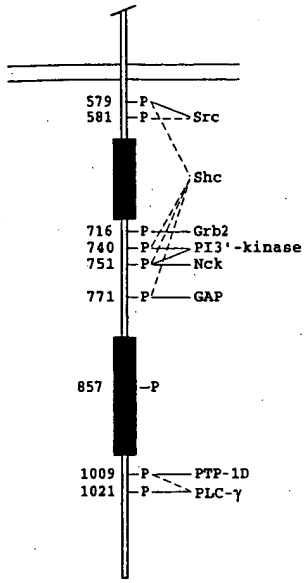

Figure 2. Interaction of SH2 Domain–Containing Signal Transduction Molecules with Different Autophosphorylation Sites in the PDGF β Receptor

Schematic illustration of the intracellular portion of a PDGF β receptor after activation. The kinase domain (closed boxes) in the receptor is divided into two parts by an inserted sequence. The tyrosine residues in the receptor known to be autophosphorylated are indicated by numbers. The interaction of individual autophosphorylated tyrosine residues with different SH2 domain–containing proteins are also indicated. Shc, Grb2, and Nck are adaptor molecules; Src denotes different members of the Src family of tyrosine kinases; PI3-kinase, phosphatidylinositol 3′-kinase; GAP, GTPase-activating protein; PTP1D, protein tyrosine phosphatase 1D; PLC-γ, phospholipase C-γ.

that a binding pocket for a phosphorylated tyrosine and the immediately surrounding amino acid residues is formed (Pawson and Schlessinger, 1993; Cohen et al., 1995 [this issue of *Cell*]). Of particular importance are the three to six amino acid residues C-terminal of the phosphorylated tyrosine (Eck et al., 1993; Pascal et al., 1994; Waksman et al., 1993); since different SH2 domains have different preferences for this region, there is specificity in the interaction. As an example, the PDGF β receptor has been shown to contain at least nine autophosphorylated tyrosine residues; Tyr-857 in the second part of the kinase domain is of importance for the catalytic activity of the kinase, whereas the others interact in a specific manner with at least eight different signal transduction molecules (reviewed by Claesson-Welsh, 1994; Figure 2).

Homodimerization or Heterodimerization

Protein-tyrosine kinase receptors are activated after homodimerization or after heterodimerization. In the case of the PDGF receptor subfamily, the different isoforms of PDGF induce different dimeric forms of the receptors. Since the A chain of PDGF binds only α receptors while the B chain binds both α and β receptors with high affinity, PDGF-AA induces αα receptor homodimers only, PDGF-AB induces αα receptor homodimers and αβ receptor heterodimers, and PDGF-BB induces all three combinations of receptors (Heldin et al., 1989; Kanakaraj et al., 1991; Seifert et al., 1989). There are certain differences in the signals transduced via αα receptor homodimers and ββ receptor homodimers, e.g., regarding the stimulation of chemotaxis and actin reorganization. Moreover, PDGF-AB, which preferentially induces αβ receptor dimers, induces a stronger mitogenic response than the other PDGF isoforms. A possible explanation for the unique properties of the αβ receptor heterodimer is the presence of unique autophosphorylation sites, not seen in the homodimeric receptors, and that may mediate interactions with additional signal transduction molecules (Rupp et al., 1994). Thus, the response to PDGF depends both on the particular isoform of PDGF and on the number of α and β receptors expressed on the target cells.

The EGF receptor was the first protein-tyrosine kinase receptor to be shown to dimerize after ligand binding (Yarden and Schlessinger, 1987). However, within the same subfamily of tyrosine kinase receptors, heterodimerization of receptors has also been observed. A candidate ligand for ErbB2 (Neu differentiation factor [NDF], also called heregulin, glial growth factor, or acetylcholin-receptor-inducing activity), which is structurally related to EGF, was found to induce heterodimeric complexes between ErbB2 and ErbB3 or ErbB4 (Peles et al., 1993; Plowman et al., 1993; Sliwkowski et al., 1994). Moreover, the presence of ErbB3 or ErbB4 was necessary for high affinity binding of NDF and signal transduction through ErbB2 to occur. Interestingly, ErbB3 lacks certain highly conserved amino acid residues in its kinase domain; consistent with this finding, ErbB3 was found to have low or no kinase activity (Prigent and Gullick, 1994). It is thus possible that the major function of ErbB3 in the heterodimer is to act as a substrate for the ErbB2 kinase and thus provide docking sites for downstream SH2 domain–containing signal transduction molecules (Carraway and Cantley, 1994); for example, binding motifs for the SH2 domains of the phosphatidylinositol 3′-kinase (PI3-kinase) are lacking in the EGF receptor and in ErbB2, but occur in several copies in ErbB3 (Fedi et al., 1994; Soltoff et al., 1994). Also, EGF itself can induce heterodimerization of EGF receptors and ErbB2 (Soltoff et al., 1994; Wada et al., 1990). In fact, heterodimerization is preferred in cells expressing both EGF receptors and ErbB2. Although heterodimerization occurred also with a kinase-inactivated ErbB2 receptor mutant, this complex was inactive, showing that in this case signaling can not occur via ErbB2 serving as a EGF receptor substrate (Qian et al., 1994).

The studies on dimerization of receptors in the PDGF receptor and EGF receptor families thus provide examples of different types of dimeric complexes induced after ligand binding, i.e., homodimeric (Figure 3A) or heterodimeric (Figure 3B) complexes between two catalytically active subunits, or a heterodimeric complex between one active and one inactive or less active subunit (Figure 3C). Given that tyrosine kinase receptors and ligands occur in families of structurally related molecules, it is not unlikely that homodimerization and heterodimerization of receptors occur in parallel also in other families, thus increasing

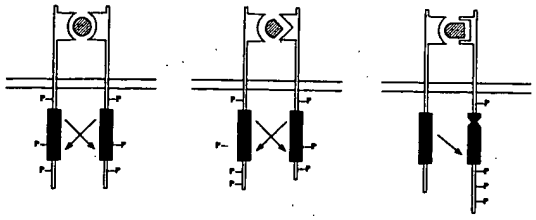

Figure 3. Different Dimeric Complexes of Protein-Tyrosine Kinase Receptors

Schematic representation of different forms of dimeric complexes of tyrosine kinase receptors formed after ligand binding. (A) a homodimeric complex; (B) a heterodimeric complex of two kinase-active subunits; (C) a heterodimeric complex of one active and one inactive or less active subunit.

the range of responses possible from a given number of receptor molecules.

One special case is the insulin and insulin-like growth factor 1 (IGF-1) receptor family. These receptors exist in the cell membrane as disulfide-bonded homo- or heterodimers of receptor subunits (each subunit is further cleaved into α and β chains by proteolysis) (Soos and Siddle, 1989). Thus, ligand binding does not induce receptor dimerization, but presumably causes a conformational alteration in the preformed dimeric receptor, which leads to receptor activation. Moreover, autophosphorylated tyrosine residues in the receptor molecules are not so important for the binding of downstream components in the signal transduction pathways; rather, the insulin receptor kinase phosphorylates insulin receptor substrate 1 (IRS-1), which mediates the interactions with SH2 domain proteins (White, 1994).

Cytokine Receptors

The cytokine receptor classes include receptors for many interleukins, colony-stimulating factors, interferons, and certain other factors and hormones (reviewed by Kishimoto et al., 1994; Mui and Miyajima, 1994; see Table 1). Class I cytokine receptors are characterized by the presence in their extracellular domains of one or two copies of a conserved domain of about 200 amino acids, which contains two modules of fibronectin type III–like motifs, four conserved cysteine residues, and the conserved motif Trp–Ser–Xaa–Trp–Ser (Bazan, 1990). Class II cysteine receptors, including receptors for interferons and interleukin-10 (IL-10), contain another conserved motif of four cysteine residues and lack the Trp–Ser–Xaa–Trp–Ser motif. The intracellular domains of cytokine receptors lack intrinsic enzymatic activities. However, despite the structural difference between cytokine receptors and tyrosine kinase receptors, their mechanism of activation appears to be similar. Ligand binding induces dimerization or oligomerization of cytokine receptors, and this allows interaction and activation of cytoplasmic protein-tyrosine kinases that are associated with the intracellular domain of the receptors.

Activation of Class I Cytokine Receptors through Formation of Hetero-Oligomeric Complexes

Most of the class I cytokine receptors undergo heterodimerization or hetero-oligomerization after ligand binding (Table 1). In many cases, the ligand-binding subunit(s) form signaling complexes with signal-transducing molecules that are structurally related to cytokine receptors, but that are themselves unable to bind ligands. For instance IL-3, granulocyte/macrophage colony-stimulating factor (GM-CSF), and IL-5 bind to specific α subunit receptors; the α subunits all interact with a common β subunit that is required for high affinity ligand binding and signal transduction (Mui and Miyajima, 1994).

Similarly, IL-6, leukemia inhibitory factor (LIF), oncostatin M, IL-11, and ciliary neurotrophic factor (CNTF) share a common signal transducer, gp130 (Taga et al., 1989); signaling is triggered by the formation of homo- or heterodimers of gp130. IL-6 binds to the IL-6 receptor and induces a complex containing a homodimer of gp130 (Murakami et al., 1993). Interestingly, signaling occurs also with a truncated IL-6 receptor lacking the cytoplasmic domain, which indicates that the IL-6 receptor is needed only to increase the binding affinty for IL-6. The CNTF receptor acts similarly, lacking a cytoplasmic domain in its natural form and being anchored in the membrane through a phosphatidylinositol group. The CNTF receptor–CNTF complex signals via formation of a heteromeric complex of gp130 and the LIF receptor (Davis et al., 1993). LIF and oncostatin M signal via binding directly to a heteromeric complex of gp130 and the LIF receptor (Gearing et al., 1992). IL-11 is dependent on gp130 but not the LIF receptor for signaling (Hilton et al., 1994).

A third subfamily is constituted by IL-2, IL-4, IL-7, and IL-9. In this family, signaling involves the formation of heterodimeric receptor complexes between specific β subunits and a common γ subunit (Kawahara et al., 1994). In the case of IL-2, the ligand binding affinity is increased by the presence also of an α subunit, which has a structure unrelated to that of cytokine receptors. Whereas the α subunit is not needed for signal transduction, both the β and the γ subunits are needed, presumably in a heterodimeric configuration (Nakamura et al., 1994; Nelson et al., 1994).

Activation by Homodimerization

Although activation by heterodimerization appears to be most common among cytokine receptors, there are examples of cytokine receptors that are activated by homodimerization, e.g., the receptors for growth hormone (GH), erythropoietin (EPO), prolactin, and granulocyte colony-stimulating factor (G-CSF) (Table 1). A well-characterized example is the GH receptor. Analysis of crystals of GH and the extracellular part of the receptor revealed that each ligand binds two receptor molecules simultaneously (Cunningham et al., 1991; de Vos et al., 1992; Ultsch et al., 1991). This finding was surprising since GH is a monomeric molecule without apparent symmetry. The two receptor-binding sites in GH are therefore different, although they bind to similar epitopes in the receptors. Site 1 is larger and is supposed to bind receptor first; the smaller site 2 thereafter binds a second receptor, and the dimeric receptor complex is further stabilized by direct interaction between the two receptors. The importance of the latter

epitope in stabilizing a GH receptor dimer is illustrated by the finding that a mutation in this region abolishes receptor homodimerization and is responsible for a form of familial GH resistance (Laron's syndrome; Duquesnoy et al., 1994). The results from the three-dimensional structure studies are supported by titration calorimetry in solution; the heat of binding was found to be saturated at a 1:2 ratio of ligand and receptor (Ultsch et al., 1991).

Signal Transduction

Much information regarding the signal transduction pathways from cytokine receptors to the nucleus has come from a genetic approach in which mutant cell lines defective in the response to interferons were isolated and characterized (Darnell et al., 1994). This approach led to the identification of three categories of proteins, a DNA-binding protein (p48), STATs (signal transducers and activators of transcription), and cytoplasmic protein-tyrosine kinases of the JAK family.

The JAK kinases are characterized by the presence of two kinase domains in each molecule, which is the basis for their name (Janus kinases, after the Roman god with two faces) (reviewed by Ziemiecki et al., 1994). Several members of this family are currently known (JAK1, JAK2, Tyk2, JAK3 [Ziemiecki et al., 1994; Takahashi and Shirasawa, 1994]); they associate in a specific manner with different cytokine receptors and are activated upon receptor dimerization. An important class of substrates for JAK kinases is members of the STAT family (STAT1α, STAT1β, STAT2, STAT3, STAT4, STAT5, and IL-4 STAT) (Darnell et al., 1994; Zhong et al., 1994; Gouilleux et al., 1994; Hou et al., 1994). After phosphorylation on tyrosine residues, the STAT molecules form homo- or heterodimers.

In the case of signaling from the interferon-α receptor, heterodimers of STAT1α (p91) or STAT1β (p84) and STAT2 (p113) are created, which move into the nucleus and form a complex with a DNA-binding protein (p48), allowing them to bind and stimulate transcription from elements in the promoters of interferon-α-induced genes (Schindler et al., 1992). Interestingly, another combination of STATs is formed after stimulation by interferon-γ, either a homodimer of STAT1α (p91) or a homodimer of STAT1β (p84) (Shuai et al., 1994; Shuai et al., 1992). These dimers do not associate with p48, but may form complexes with other related proteins. Both homodimers bind to interferon-γ-activated sites (GAS), which are present in interferon-γ-inducible genes, although only the STAT1α homodimer activates transcription (Shuai et al., 1993).

It thus appears that the activities of STATs are regulated by specific assembly into homo- or heterodimers. The dimerization is triggered by phosphorylation. A single phosphorylated tyrosine residue has been identified in STAT1 after stimulation with interferon-α as well as after stimulation with interferon-γ; mutation of this tyrosine residue to a phenylalanine residue prevents dimerization (Shuai et al., 1993). Since STATs contain SH2 domains, it is likely that the dimerization involves reciprocal interactions between the SH2 domains and the tyrosine-phosphorylated regions in the STAT molecules (Shuai et al., 1994). How is the specificity regulated? One possibility is that STATs may associate in a differential manner with different receptors (Fu and Zhang, 1993; Greenlund et al., 1994).

Another possibility is that the JAK family members differ in their substrate specificities and thus phosphorylate different STAT molecules. Interestingly, the genetic approach led to the identification of different JAK kinases in the signaling pathways of interferon-α (JAK1 and Tyk2) and interferon-γ (JAK1 and JAK2) (Müller et al., 1993; Velazques et al., 1992; Watling et al., 1993). Thus, in each case, there was a need for two different JAK kinases. It is unlikely that the two kinases are needed in a sequential activation mechanism, since in cells deficient in JAK1 no activation of JAK2 was seen after stimulation with interferon-γ, or vice versa (Müller et al., 1993). Thus, it is possible that the active forms of the JAK kinases involved in the signal pathways of interferons are activated by heteromeric interactions, possibly involving cross-phosphorylations.

A possible mechanism to achieve such heterodimerization is via ligand-dependent formation of heteromeric receptor complexes. The receptor for interferon-γ consists of at least two different chains (Aguet et al., 1988; Hemmi et al., 1994; Soh et al., 1994), and it has been suggested that JAK1 and JAK2 interact with these chains in a differential manner (Greenlund et al., 1994). An interferon-α/β receptor that binds JAK1 has been identified (Novick et al., 1994); whether another receptor subunit with affinity for Tyk2 exists remains to be elucidated. An analogous situation appears to prevail for the IL-2 receptors; the β and γ subunits have been shown to bind JAK1 and JAK3, respectively (Miyazaki et al., 1994; Russell et al., 1994).

Common and Unique Signals

In addition to the receptors for interferon-α and interferon-γ, many other receptors, including GH, EPO, prolactin, G-CSF, LIF, gp130, the common β subunit for the IL-3 subfamily of receptors, and the common γ subunit for the IL-2 subfamily, have been shown to bind different members of the JAK family (Ihle et al., 1994). JAKs bind in a specific manner to conserved regions called box 1 and box 2 regions in the juxtamembrane parts of cytokine receptors (Murakami et al., 1991).

Other signal transduction pathways are also initiated at the activated cytokine receptor complexes; these pathways are dependent on more C-terminal regions in the receptors. For instance, members of the Src family of kinases bind to the C-terminal tail of the IL-2 β receptor (Hatakeyama et al., 1991) and to gp130 (Ernst et al., 1994). Moreover, whereas JAK kinases bind to the juxtamembrane part of the common β subunit of IL-3, IL-5, and GM-CSF, deletion of the C-terminus abrogates Shc phosphorylation, Ras activation, and induction of c-*fos* and c-*jun* (Sato et al., 1993). Likewise, a region C-terminal of the JAK kinase–binding site of the G-CSF receptor mediates induction of granulocyte-specific genes (Fukunaga et al., 1993).

The fact that certain receptor subunits/signal transducers are shared by several cytokines, as well as the fact that different receptors may bind and activate the same JAK kinases and possibly also share other signal transduc-

tion molecules, provides an explanation for the functional redundancy and pleiotropy of different cytokines. Conversely, the presence of unique epitopes in receptors or receptor combinations may allow the transduction of specific signals that mediate unique properties of the different cytokines.

Sharing of Signal Transduction Pathways between Tyrosine Kinase Receptors and Cytokine Receptors

There is no sharp division in the modes of signaling between tyrosine kinase receptors and cytokine receptors. EGF and PDGF, acting via tyrosine kinase receptors, induce the phosphorylation of STAT1α, perhaps directly or via activation of JAK kinases (Fu and Zhang, 1993; Ruff-Jamison et al., 1993; Sadowski et al., 1993; Silvennoinen et al., 1993). Moreover, after activation of cytokine receptors, JAK kinases or possibly other kinases phosphorylate the cytokine receptors themselves. This gives SH2 domain–containing signal transduction molecules the possibility to interact with the cytokine receptors and initiate pathways initially identified for tyrosine kinase receptors, e.g., leading to activation of Ras and PI3-kinase (Boulton et al., 1994; Mui and Miyajima, 1994).

Antigen Receptors

The T cell receptor is composed of transmembrane proteins with very short cytoplasmic sequences, which are associated with a large number of invariant subunits also lacking intrinsic enzymatic activities, but capable of interacting with cytoplasmic tyrosine kinases (Figure 1) (for reviews see Cambier and Jensen, 1994; Weiss and Littman, 1994). The invariant subunits (γ, δ, ε, ζ, and η chains) contain one to three copies of a conserved 26 amino acid motif of pairs of tyrosine and leucine residues (Reth, 1989), called the antigen recognition activation motif (ARAM; also called tyrosine-based activation motif, or antigen receptor homology 1). Tyrosine kinases of the Src family bind to the T cell receptor even in the resting state. In conjunction with receptor activation, the tyrosine residues in the ARAMs are phosphorylated, presumably by Src family kinases (in T cells primarily Lck). This gives another tyrosine kinase, ZAP-70, which has two SH2 domains, the opportunity to bind to the phosphorylated sites, after which it becomes activated by phosphorylation on tyrosine residues, most likely also by Src family tyrosine kinases (Iwashima et al., 1994; Letourneur and Klausner, 1992). The precise mechanism that triggers antigen receptor activation and phosphorylation of the ARAM sequences is not known, although a possible scenario is that antigen binding causes receptor aggregation that makes possible interactions and cross-phosphorylation of tyrosine kinases in the Src family. Consistent with this possibility are the observations that chimeric molecules consisting of cytoplasmic parts of ARAM–containing T cell receptor subunits and extracellular domains of other molecules mediate activation of T cells after cross-linking (Letourneur and Klausner, 1992; Irving and Weiss, 1991; Romeo and Seed, 1991).

The B cell receptor and Fc receptors also occur in complexes containing signal transducing molecules with ARAMs, suggesting similar mechanisms of signal transduction (Clark et al., 1994; Law et al., 1993; Ravetch, 1994). Interestingly, a sequential activation of Src family members and ZAP-70/Syk family members may also be involved in cytokine signaling. The G-CSF receptor has been shown to be associated with Lyn, a member of the Src family; after stimulation, an ARAM-like motif in the C-terminus of the G-CSF receptor is phosphorylated, which binds Syk leading to its activation (Corey et al., 1994).

TNF Receptor Family

An interesting variation on the "activation by oligomerization" theme is provided by members of the TNF receptor family, which are involved in regulation of cytotoxicity, apoptosis, and proliferation (for reviews see Bazan, 1993; Smith et al., 1994). TNF occurs as two forms, TNFα and TNFβ, which both binds to two different receptors, TNF receptor 1 and 2 (p55 and p75, respectively). The TNFs are nondisulfide-bonded trimers, and elucidation of the X-ray structure of TNFβ and TNF receptor 1 (Banner et al., 1993) revealed that ligand binding induces trimerization of the receptor. Each TNF subunit makes contact with two adjacent receptor molecules, thus stabilizing the receptor trimer. It is likely that the activating event is receptor aggregation, but it is not clear whether there is a need for receptor trimerization, or whether receptor dimerization would be sufficient for activation. In support of the possibility that trimerization of TNF receptor 1 is, in fact, necessary for signal transduction, monoclonal antibodies against this receptor, which are expected to dimerize the receptor, do not lead to activation, whereas activation occurs after cross-linking of the monoclonals with a second antibody, or after stimulation by two monoclonals directed against different epitopes (Engelmann et al., 1990).

A novel family of molecules that associate with the cytoplasmic part of TNF receptor 2 and that may serve as signal transducers was recently identified (Rothe et al., 1994); TNF receptor associated factors, TRAF1 and TRAF2, contain a novel region of homology and form homo- or heterodimers. This finding represents an important step in the understanding of signaling from the TNF receptor 2, but the mode of activation of TRAFs, their downstream effectors, and whether related molecules are involved in signaling from other members in the TNF receptor family remain to be elucidated.

Protein-Serine/Threonine Kinase Receptors

Transforming growth factor β (TGFβ) is a prototype for a large family of structurally related factors that regulate cell growth and differentiation, including in addition to TGFβs, e.g., activins and inhibins, bone morphogenic proteins, and Müllerian inhibition substance. As far as has been characterized, these molecules exert their cellular effects by binding to heteromeric complexes of serine/threonine kinase receptors (reviewed by Massagué et al., 1994; Miyazono et al., 1994).

Both type I and type II receptors have rather small cysteine-rich extracellular domains; the type I receptors, which are more similar to each other than to the type II receptors, all have a characteristic region rich in glycine and serine

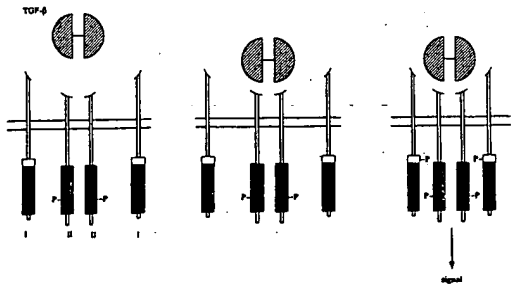

Figure 4. Signaling via TGFβ Receptors

Schematic illustration of the mechanism of signaling via TGFβ receptors as suggested by Wrana et al. (1994). TGFβ binds first to type II receptors that have a constitutively active serine/threonine kinase. The type I receptor is then incorporated in the complex and activated by phosphorylation in the GS box. Serine/threonine kinase domains are stippled, and GS boxes are open.

residues (GS domain) in their cytoplasmic juxtamembrane domains. Both receptors are needed for signaling (Wrana et al., 1992), and the cytoplasmic parts of the receptors are not interchangeable (Okadome et al., 1994). Ligand binding induces a hetero-oligomeric complex of type I and type II receptors, most likely a heterotetramer containing two receptors of each type (Yamashita et al., 1994). Studies on TGFβ-induced phosphorylation of the receptor molecules have led to an interesting model for activation of the receptors (Wrana et al., 1994; Figure 4). The type II receptor, which occurs in a dimer also in the absence of ligand (Henis et al., 1994; Chen and Derynck, 1994) and has a constitutively active kinase, first binds TGFβ. This complex then recruits the type I receptor, which can not bind ligand in the absence of type II receptor, resulting in the phosphorylation of the type I receptor on serine residues in the GS domain. The phosphorylation presumably activates the type I receptor kinase that now can act on downstream components in the signal transduction pathway. Other members in the TGFβ family also form heteromeric complexes containing different members of the type I and type II receptor subfamilies. Thus, sequential phosphorylation between the type II and type I receptors may be a general mechanism of receptor activation of members of the serine/threonine kinase receptor family.

Is Dimerization Sufficient for Activation?

There are several examples in which activation of receptors occurs after dimerization or oligomerization induced by means other than ligand binding. Many tyrosine kinase receptors, for instance, are activated after binding of antibodies, whereas Fab fragments generally are inactive. Insertion of an extra cysteine residue in the extracellular juxtamembrane region of the EGF receptor led to the formation of a constitutively active dimeric receptor (Sorokin et al., 1994). Moreover, mutated forms of many of the tyrosine kinase receptors have been identified as transforming oncogenes. In some cases, the activating mechanism is a gene rearrangement that leads to the production of a fusion protein between a novel protein and the kinase domain of the receptor. The fusion partners are often domains of proteins that undergo oligomerization in their normal context. Examples include tropomyosin, which has been found fused to Trk (Martin-Zanca et al., 1986), the regulatory subunit of the cyclic AMP-dependent protein kinase, which has been found fused to Ret (Takahashi et al., 1985), and sequences from Tpr, containing a leucine zipper, which has been found fused to Met (Park et al., 1986; Rodrigues and Park, 1994) as well as to Trk (Greco et al., 1992). Another mechanism is exemplified by the Neu (ErbB2) oncogene product, which obtained transforming activity by a single amino acid exchange in the transmembrane region that promotes receptor aggregation (Weiner et al., 1989). In these cases, artificially induced receptor dimerization leads to activation of the kinase domains and autophosphorylation in a ligand-independent manner.

Also cytokine receptors can acquire transforming properties after mutation. A constitutively active EPO receptor mutant was found to have an arginine residue replaced with a cysteine residue in a region corresponding to the receptor dimer interface of the related GH receptor; this resulted in the formation of a disulfide bond that stabilized the receptor dimer in a ligand-independent manner (Watowich et al., 1992). This finding further supports the concept that dimerization is sufficient for activation of many receptor types.

Antagonists

There are many examples of tyrosine kinase receptors and cytokine receptors that after mutations in their cytoplasmic domains act in a dominant negative manner, i.e., when expressed in cells with the corresponding wild-type receptor, they attenuate the signals induced by ligands. The mechanism for the dominant negative effect is that the wild-type receptors after ligand binding are locked up in sterile heteromeric complexes with the mutated receptors. These findings provide support for the notion that dimerization of wild-type receptors is necessary for activation of many receptor types; however, alternative modes for activation have not been excluded. Another way in which the oligomerization process can be antagonized is through mutated versions of certain ligands. For example, mutation of one of the two receptor-binding sites in GH yielded a GH protein with antagonistic properties (Fuh et al., 1992). Moreover, mutation of a glutamic acid residue in GM-CSF (Glu-21) that is important for the interaction with the common β subunit (Hercus et al., 1994), mutation of Tyr-124 in IL-4, which is important for interaction with the common γ subunit (Kruse et al., 1992), or mutation of Tyr-31 and Gly-35 in IL-6, which are important for interaction with the gp130 signal transducer (Savino et al., 1994), yielded molecules with antagonistic effects in their respective systems.

It is possible that inhibition of receptor oligomerization is a generally applicable method to antagonize growth factor and cytokine action. Antagonistic ligands and antibodies may have particular clinical utility in conditions of overactivity of growth factors and cytokines, since they can act specifically.

Conclusions

It is now well established that several receptor types are activated through ligand-induced receptor dimerization or oligomerization. Dimerization combines accuracy with flexibility; there is specificity in binding of the ligand to the receptors and flexibility in the assembly of different homo- or heterodimeric receptor subunits depending on which receptors and signal transducers are expressed by a particular cell. There are also examples of receptors that do not dimerize after ligand binding, e.g., the serpentine receptor family, which transverses the cell membrane seven times and couples to G proteins, and ion channel receptors. However, for receptor molecules that are anchored in the membrane with a single transmembrane domain, dimerization or oligomerization may be a general mechanism for receptor activation.

A general feature of receptors generating growth stimulatory signals seems to be activation of tyrosine kinases in the receptor complex. Although the exact mechanisms for activation of the kinases remain to be elucidated, interactions and cross-phosphorylations between identical or related kinases induced by receptor dimerization are common. The resulting phosphorylations of tyrosine residues on receptor and signal transducing components trigger interactions with SH2-containing molecules (see Cohen et al., 1995). Growth inhibitory signals from the activated TGFβ receptor complex involve phosphorylation on serine/threonine residues in yet unknown substrates. Thus, much of intracellular signaling is regulated by phosphorylation events. To understand the regulation of signal transduction, it will therefore be important to characterize not only the kinases involved, but also the phosphatases that counteract the effects of kinases (see Hunter, 1995 [this issue of *Cell*]).

Acknowledgments

I thank my colleagues in Uppsala and Joseph Schlessinger for valuable comments and suggestions and Ingegärd Schiller for skillful secretarial assistance. I apologize for not having been able to cite all relevant literature, because of journal policy limiting the length of the reference list.

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The EMBO Journal vol.14 no.22 pp.5569–5578, 1995

Exhibit 3

Point mutations within a dimer interface homology domain of c-Mpl induce constitutive receptor activity and tumorigenicity

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c-Mpl, a receptor for thrombopoietin (TPO), belongs to the haemopoietin/cytokine receptor superfamily, a group of cell surface molecules characterized by conserved sequence motifs within their ligand binding domains. A recurring mechanism for the activation of haemopoietin receptors is the formation of functional complexes by receptor subunit oligomerization. Within the growth hormone receptor, a cluster of extracellular amino acids forms a dimer interface domain that stabilizes ligand-induced homodimers. This domain appears to be functionally conserved in the erythropoietin (EPO) receptor because substitution of cysteines for residues in the analogous region causes EPO-independent receptor activation via disulfide-linked homodimerization. This report identifies an homologous domain within the c-Mpl receptor. The substitution of cysteine residues for specific amino acids in the dimer interface homology regions of c-Mpl induced constitutive receptor activity. Factor-dependent FDC-P1 and Ba/F3 cells expressing the active receptor mutants no longer required exogenous factors and proliferated autonomously. The results imply that the normal process of TPO-stimulated Mpl activation occurs through receptor homodimerization and is mediated by a conserved haemopoietin receptor dimer interface domain. Moreover, cells expressing activated mutant Mpl receptors were tumorigenic in transplanted mice. Thus, like v-*mpl*, its viral counterpart, mutated forms of the cellular *mpl* gene also have oncogenic potential.

Keywords: c-*mpl*/haemopoietin receptor/tumorigenicity

Introduction

The c-*mpl* gene was discovered as the cellular homologue of v-*mpl*, the oncogene of the murine myeloproliferative leukaemia virus (MPLV; Souryi *et al.*, 1990; Vigon *et al.*, 1992). In mice, v-*mpl* induces a lethal myeloproliferative disease characterized by the rapid appearance of factor-independent haemopoietic progenitors and an acute leukaemia involving multiple haemopoietic lineages (Wendling *et al.*, 1986, 1989). The cellular gene encodes a cell surface receptor that is expressed predominantly in primitive haemopoietic cells, megakaryocytes and platelets (Souryi *et al.*, 1990; Vigon *et al.*, 1992; Methia *et al.*, 1993; Debili *et al.*, 1995). Consistent with this expression pattern, the ligand for c-Mpl has been identified recently as thrombopoietin (TPO, also referred to as Mpl ligand or megakaryocyte growth and development factor; Bartley *et al.*, 1994; de Sauvage *et al.*, 1994; Lok *et al.*, 1994). In culture, recombinant TPO stimulates the proliferation of megakaryocyte progenitors (CFU-Mk) and induces maturation of megakaryocytes. Its administration to mice also stimulates CFU-Mk production, elevates megakaryocyte numbers in the bone marrow and spleen and significantly increases the number of circulating platelets (de Sauvage *et al.*, 1994; Kaushansky *et al.*, 1994; Broudy *et al.*, 1995). The c-Mpl receptor is clearly essential for normal thrombopoiesis. Reagents that neutralize TPO activity prevent normal megakaryocyte development *in vitro* (Wendling *et al.*, 1994; Kaushansky *et al.*, 1995), and mice in which c-*mpl* has been disrupted via homologous recombination in ES cells produce only 10–20% of the normal number of megakaryocytes and platelets (Gurney *et al.*, 1994).

c-Mpl belongs to the haemopoietin or cytokine receptor superfamily, members of which share a conserved extracellular haemopoietin receptor domain defined by four evenly spaced cysteine residues, series of alternating hydrophobic and polar residues and the distinctive Trp–Ser–Xaa–Trp–Ser (WSXWS) motif (Gearing *et al.*, 1989; Bazan, 1990; Cosman *et al.*, 1990). A recurring theme within this family is receptor subunit oligomerization, which characterizes the process of ligand-stimulated receptor activation. Many haemopoietin receptors form active, high affinity complexes through the hetero-oligomerization of ligand-specific α-chains with shared signal transducing β-chains. Interleukin (IL)-2, IL-4, IL-7, IL-9 and IL-13 each bind a specific receptor chain and recruit a common component, the IL-2 receptor γ-chain, to the active complex (reviewed in Taniguchi and Minami, 1993; Kishimoto *et al.*, 1994). Similarly, IL-3, IL-5 and granulocyte–macrophage colony-stimulating factor bind specific receptors at low affinity and then interact with the shared β_c chain at high affinity for signal transduction (reviewed in Nicola and Metcalf, 1991; Miyajima *et al.*, 1993). Finally, the receptors for IL-6, IL-11, leukaemia inhibitory factor (LIF), oncostatin M and ciliary neurotropic factor form active complexes which combine α-chains with the LIF receptor and/or gp130 (Hibi *et al.*, 1990; Gearing *et al.*, 1992; Ip *et al.*, 1992; Davis *et al.*, 1993; Hilton *et al.*, 1994). A distinct subset of the haemopoietin receptors form homodimers following ligand binding, including those for growth hormone (GH), prolactin and granulocyte colony-stimulating factor (G-CSF; Elberg *et al.*, 1990; Fukunaga *et al.*, 1990; Cunningham *et al.*, 1991; Fuh *et al.*, 1992; Hooper *et al.*, 1993). The erythropoietin (EPO) receptor appears also to be activated in this manner, as mutation of specific amino acids to cysteine residues

within the extracellular domain forces disulfide-linked homodimerization and results in constitutive, EPO-independent activation (Yoshimura *et al.*, 1990; Watowich *et al.*, 1992, 1994).

The crystal structure of the GH–GH receptor complex provides a model for haemopoietin receptor structure and subunit interaction. The haemopoietin domain consists of two subdomains, each composed of seven β-strands which form a sandwich of two antiparallel β-sheets (de Vos *et al.*, 1992). The GH–GH receptor complex, which contains a GH receptor homodimer bound by a single hormone molecule, is stabilized not only by ligand–receptor interactions but also through intermolecular interaction of amino acids located primarily within the same membrane-proximal domain of each receptor monomer. Significantly, the region of the EPO receptor that is sensitive to activation by cysteine substitution mutation aligns with the domain of the GH receptor most involved in dimer stabilization (Watowich *et al.*, 1994). These receptors therefore appear to share a structurally conserved dimer interface domain.

To explore the biochemical mechanisms and biological consequences of c-Mpl activation, we have generated constitutively active receptor mutants. We report here the identification of dimer interface homology regions within the c-Mpl receptor in an analogous position to those conserved between the GH and EPO receptors. Substitution of cysteine residues into this domain, designed to promote disulfide-bonded homodimerization, constitutively activated Mpl: factor-dependent cells expressing these mutants no longer required exogenous factors for growth and became tumorigenic. The results imply that the normal process of TPO-induced c-Mpl activation involves receptor homodimerization mediated by a dimer interface domain that is conserved among other haemopoietin receptor family members. Moreover, the tumorigenicity of cells expressing activated *mpl* mutants reveals that, like its viral counterpart, the cellular *mpl* gene is capable of contributing to oncogenesis.

Results

The amino acid sequences of the GH, EPO and c-Mpl receptor haemopoietin domains were compared using the conserved cysteine pairs, stretches of alternating hydrophobic and polar residues and the WSXWS motifs as major conserved landmarks and aligning the intervening residues for best homology. Figure 1A shows a localized region of this comparison around the dimer interface domain of the human GH receptor, which aligns with the region of the EPO receptor into which the introduction of cysteine residues causes constitutive dimerization and receptor activation (see also Watowich *et al.*, 1994). To determine whether activation of the c-Mpl receptor involves an analogous domain, residues in the dimer interface homology region of each of the two Mpl haemopoietin domains were mutated to cysteine. Codons 117 (*mpl*R117C) and 120 (*mpl*S120C) in the N-terminal haemopoietin domain, and 368 (*mpl*S368C) and 369 (*mpl*S369C) in the membrane-proximal domain were altered individually by site-directed mutagenesis to encode cysteine residues (Figure 1). Each mutant *mpl* cDNA, as well as the wild-type sequence, was inserted into the LXSN retroviral vector to allow expression in cells via viral infection (see Materials and methods).

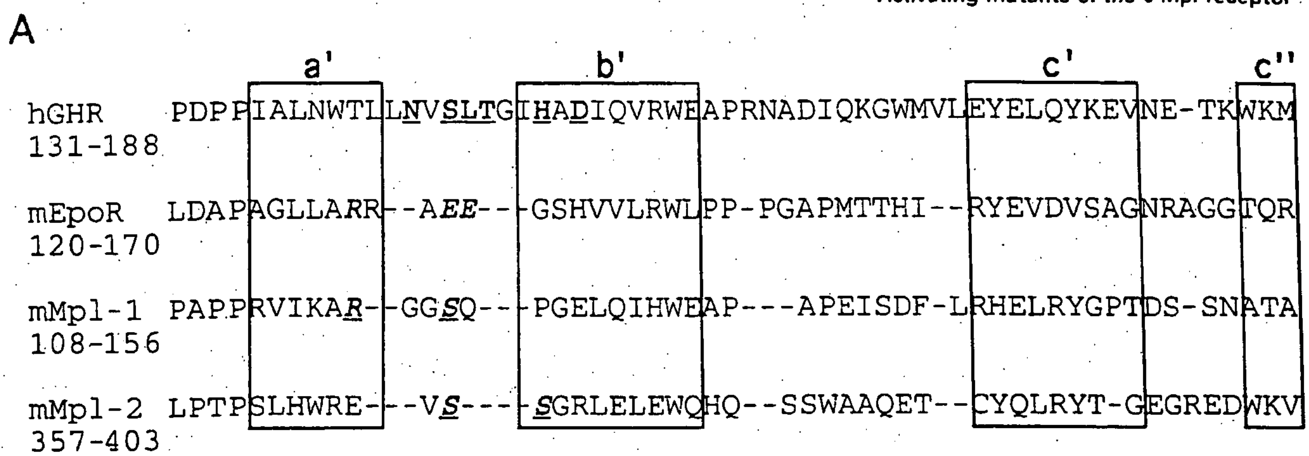
Activity of c-mpl mutants

The activity of each *mpl* mutant was assessed by determining the capacity of the altered receptors to produce a proliferative signal in factor-dependent FDC-P1 and Ba/F3 cells. Parental (uninfected) cells were strictly dependent on exogenous IL-3 for survival and growth and did not produce colonies in agar in the absence of added factor (Figure 2). Upon infection with the LXSN virus or its wild-type (wt) or mutant *mpl* derivatives, each of which also carries the neo^R gene, the cells gained the capacity to grow in the cytotoxic drug G418, confirming that they had been productively infected (Figure 2). However, only cells infected with mutant *mpl* viruses acquired the ability to produce agar colonies in the absence of exogenous growth factors. Relatively high numbers of factor-independent colonies consistently arose from *mpl*S368C virus-infected FDC-P1 or Ba/F3 populations. Smaller numbers were observed in *mpl*S369C cultures, and only sporadic colonies arose from *mpl*R117C virus-infected cells. The *mpl*S120C mutant consistently failed to stimulate autonomous cell growth, as did the *mpl*wt receptor, as would be expected in the absence of ligand (Figure 2).

The ligand-independent activity of mutant Mpl receptors demonstrates that the substitution of cysteine residues for amino acids within a conserved dimer interface homology domain does indeed constitutively activate receptor function. To estimate more quantitatively the efficiency with which each mutant conferred factor independence, Ba/F3 cells were infected with the wt or mutant *mpl* viruses and selected for receptor expression in liquid cultures with a maximal concentration of TPO as the sole stimulus. Cells proliferated in all cultures, confirming that each of the *mpl* mutants was appropriately expressed and the proportion of autonomous cells in each population was then measured in agar cultures. As suggested in the initial experiments, the *mpl*S368C mutant most efficiently induced factor independence: 48% of cells selected to express sufficient *mpl*S368C to respond to TPO were capable of autonomous growth (Figure 3A). Only 7% of TPO-responsive Ba/F3-*mpl*S369C cells were factor-independent and no autonomous colonies arose from 10^3 receptor-expressing Ba/F3-*mpl*R117C, -*mpl*S120C or -*mpl*wt cells (Figure 3A). As expected, control cultures of uninfected or Ba/F3-LXSN cells failed to proliferate in TPO, as parental cells lack endogenous Mpl receptors. The dose–response of Ba/F3 cells expressing *mpl*R117C or *mpl*S120C to TPO was similar to that of Ba/F3-*mpl*wt cells in proliferation assays (Figure 3B). Thus, the inactivity or low efficiency of these mutants in stimulating autonomous growth do not appear to stem from poor expression or defective transport to the cell surface, nor from any intrinsic defect in signal transduction resulting from the cysteine substitution mutations.

Establishment of factor-independent clones

Factor-independent colonies from primary agar cultures of mutant *mpl* virus-infected cells were selected for expansion as clones in liquid culture. Essentially all colonies picked from FDC-P1 cultures expressing *mpl*R117C, *mpl*S368C or *mpl*S369C continued to prolifer-



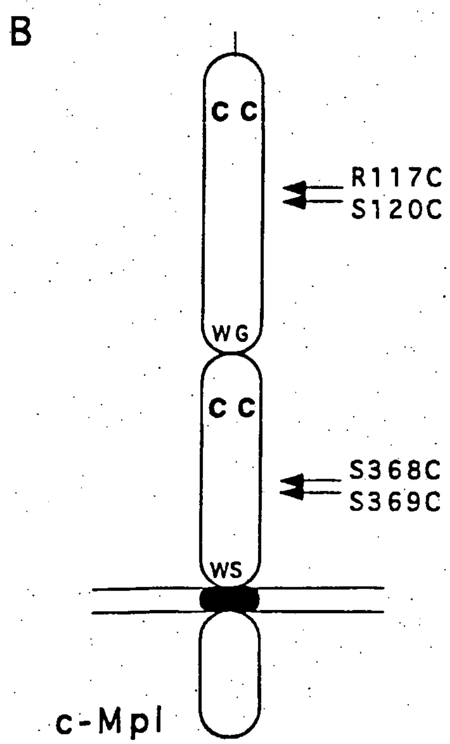


Fig. 1. (A) Amino acid sequence alignment of part of the haemopoietin receptor domains of the human growth hormone (hGHR), and murine EPO (mEPOR) and c-Mpl receptors. The region shown encompasses the GH receptor dimer interface domain (with residues involved in salt bridges or hydrogen bonds between receptor monomers underlined) and the domain of the EPO receptor where alteration of particular residues (italicized) to cysteines induces constitutive activation. The homologous region of both the N-terminal (mMpl-1) and membrane-proximal (mMpl-2) c-Mpl haemopoietin domains is also aligned, with residues targeted for mutation in this study both italicized and underlined. The region aligned from each receptor is indicated by amino acid number. The boxed sequences are regions of β-sheet structure (designated a', b', c' and c'') derived from the GH receptor crystal structure and predicted to exist in other haemopoietin receptors (Bazan, 1990; de Vos *et al.*, 1992).
(B) Schematic representation of the c-Mpl receptor indicating the conserved cysteine residues (CC) and WSXWS motifs in each of the two haemopoietin domains and with the transmembrane region shaded. The positions of the amino acids changed to cysteines in this paper are indicated with arrows.

ate autonomously as did those from Ba/F3-*mpl*S368C and -*mpl*S369C cultures (Table I). Over several experiments, only one colony grew from Ba/F3-*mpl*R117C cells in the absence of added factors and it subsequently failed to expand when transferred into liquid culture. From both FDC-P1 and Ba/F3 cells, all colonies that expanded initially continued to proliferate upon further passage and were established as factor-independent cell lines.

To confirm the origin of these autonomous cell lines, genomic DNA was analysed by Southern blotting for retroviral integration. Proviruses containing the c-*mpl* cDNA were detected in all clones analysed, as shown for several examples in Figure 4. Using unique restriction endonucleases engineered into each mutant cDNA (see Materials and methods), the origin of each clone from the appropriate mutant *mpl* virus was also confirmed (data not shown). Interestingly, the number of proviral integrations was on average twice as high in FDC-P1- or Ba/F3-*mpl*S369C clones than in their *mpl*S368C counterparts (Figure 4 and Table I). The relative inefficiency of the S369C mutant in comparison with S368C in stimulating autonomous growth (Figure 3A) may demand higher expression levels resulting in selection of clones with multiple integrated proviruses (see Discussion).

Factor-independent proliferation of mplS368C cells is density independent

The growth properties of several clones of FDC-P1 and Ba/F3 cells expressing the constitutive *mpl*S368C mutant were analysed in detail. In addition to proliferating autonomously in liquid cultures and carrying integrated *mpl*S368C proviruses, Northern blot analysis was used to confirm that these clones also expressed the appropriate exogenous *mpl* transcripts (data not shown). The number of colonies formed in agar per cell plated (cloning efficiency) was 65–85% for FDC-P1-*mpl*S368C clones and 30–50% for Ba/F3-*mpl*S368C clones. The cloning efficiency of all clones was independent of the number of cells plated. Moreover, the addition of maximal concentrations of WEHI-3BD$^-$-conditioned medium or TPO did not consistently influence the efficiency of colony formation (Figure 5). Media harvested from 5 ml cultures of each

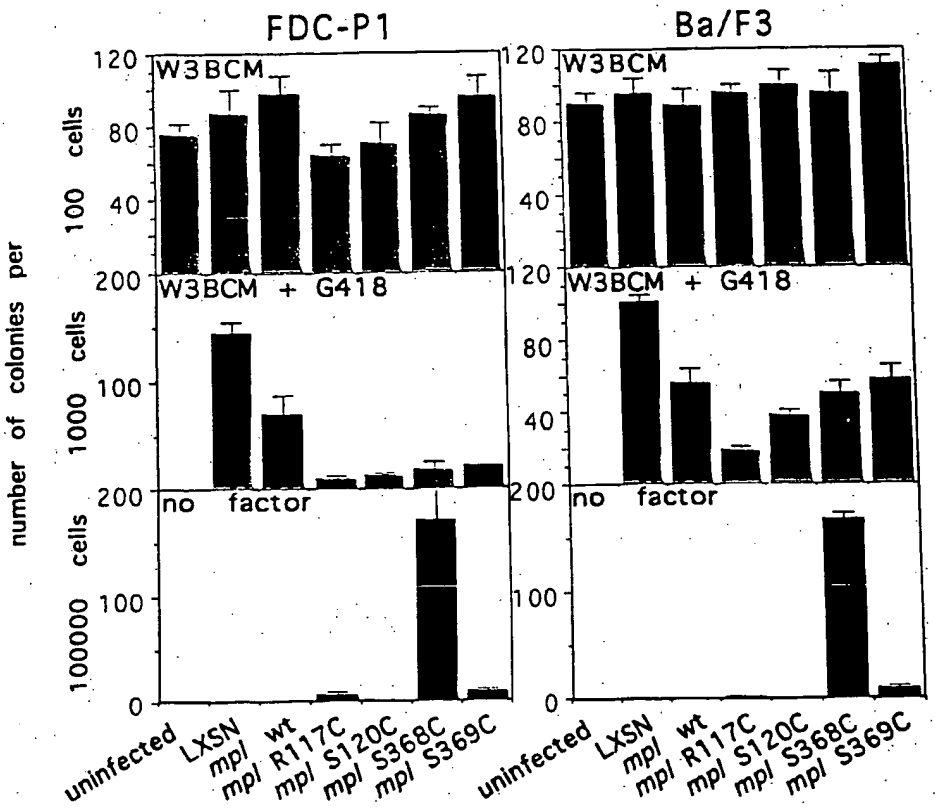


Fig. 2. Colony formation in agar cultures by uninfected FDC-P1 and Ba/F3 cell populations and following their infection with the LXSN virus or viruses expressing wild-type (wt) or cysteine-mutant (R117C, S120C, S368C, S369C) Mpl receptors. The bars show the mean number of colonies stimulated by WEHI3B-D$^-$-conditioned medium (W3BCM), a source of IL-3, in the presence or absence of the cytotoxic drug G418 or in unstimulated (no factor) cultures. Error bars indicate the standard error of the mean of triplicate determinations.

of three FDC-P1- or Ba/F3-*mpl*S368C clones 24–48 h after seeding at 5×10^5 cells/ml were assayed for the production of growth factors. None of the clones secreted factors capable of stimulating Ba/F3 cells expressing wt Mpl receptors, even when the conditioned medium was concentrated 10-fold (data not shown). The density-independence of cell proliferation and the absence of detectable secreted stimuli suggest that the mutant MplS368C receptor induces factor independence in the absence of any apparent autocrine mechanism.

The role of disulfide-bonded dimerization in constitutive MplS368C activity

The substitution of cysteine residues into c-Mpl was designed to induce constitutive receptor activity through disulfide-linked homodimerization. To examine the structure of mutant Mpl receptors in factor-independent cells, Ba/F3 cell clones were derived that expressed normal (*mpl*wt-F) or S368C mutant (*mpl*S368C-F) Mpl receptors containing an epitope tag at their N-terminus. The epitope-tagged receptors exhibited identical properties to their untagged counterparts: Ba/F3-*mpl*wt-F cells responded to TPO but failed to grow in the absence of exogenous factors and Ba/F3-*mpl*S368C-F cells were autonomous (data not shown). Receptor protein was precipitated from cell lysates with an antibody directed against the epitope tag, separated in SDS–polyacrylamide gels and Western blotted with the same antibody (see Materials and methods). As shown in Figure 6A, both Ba/F3-*mpl*wt-F and factor-independent Ba/F3-*mpl*S368C-F cells abundantly expressed a protein that was absent in uninfected cells and was the size expected of the c-Mpl receptor (Skoda *et al.*, 1993). Consistent with the presence of disulfide-linked Mpl homodimers, significant levels of a protein species which migrated at a molecular weight approximately twice that of c-Mpl and that reduced to monomeric size upon treatment with 2-mercaptoethanol (2-ME), were precipitated from Ba/F3-*mpl*S368C cells (Figure 6A). A larger species that may have resulted from additional receptor aggregation was also observed in these cells under non-reducing conditions.

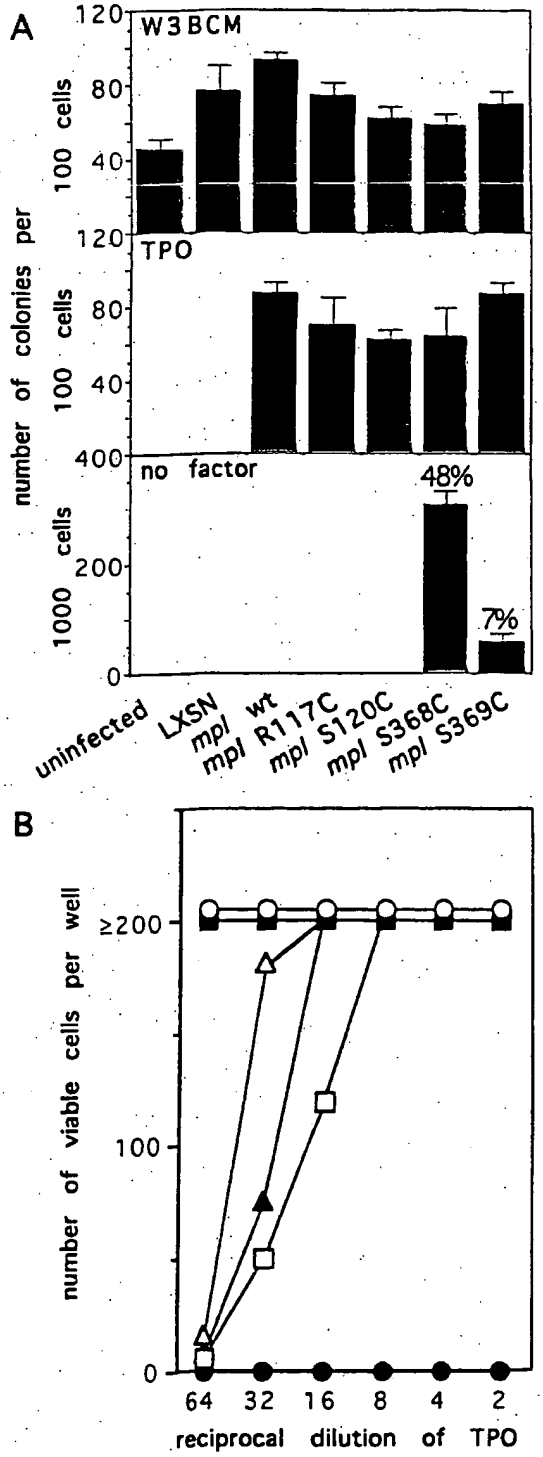
The proliferation of factor-independent cells was also assessed in the presence of chemical reducing agents. At appropriate concentrations of 2-ME, the reduced form of glutathione or α-monothioglycerol (α-MTG), factor-independent growth of FDC-P1-*mpl*S368C cells was inhibited (Figure 6B), consistent with dependence on disulfide bond formation for receptor activity. In the presence of TPO, when the cells no longer depend on constitutive receptor activity, proliferation was unaffected by the presence of reducing agents (Figure 6B). Similar results were obtained with Ba/F3-*mpl*S368C cells (data not shown). With the protein studies above, these data strongly imply that MplS368C receptors form disulfide-linked dimers and that this dimerization is critical for constitutive receptor activity.

Cells expressing mplS368C are tumorigenic

When 10^6 cells of each of three FDC-P1-*mpl*S368C clones were injected subcutaneously into syngeneic DBA/2 mice, tumours became apparent at the site of injection within 2–4 weeks. No mice injected with parental factor-dependent

FDC-P1 cells developed tumours within a 90 day observation period (Table II). In addition to a subcutaneous tumour at the site of injection, FDC-P1-*mpl*S368C cells also induced considerable splenomegaly and occasional enlargement of local lymph nodes in transplanted animals. Several mice also exhibited extensive intra-abdominal tumours and ascites fluid. Ba/F3-*mpl*S368C cells exhibited a somewhat weaker tumorigenic phenotype. Tumours arose only in irradiated (500 rad) recipient mice and not all injected mice succumbed. Nevertheless, the mutant MplS368C receptor clearly predisposed Ba/F3 cells to tumorigenicity, as uninfected cells or those expressing normal Mpl receptors were not tumorigenic (Table II). The majority of tumours arising in Ba/F3-*mpl*S368C-transplanted mice developed at the injection site without obvious splenomegaly or involvement of other organs.

A proviral integration pattern identical to that of the injected cells was detected in DNA from subcutaneous tumour or ascites samples taken from each tumour-bearing mouse (data not shown), confirming that the tumours originated from the appropriate autonomous FDC-P1- and Ba/F3-*mpl*S368C clones. Thus, constitutive receptor activation induced by mutation reveals that the cellular *mpl* gene, like its v-*mpl* counterpart, can contribute to tumorigenesis (see Discussion).



Discussion

An homologous domain within two members of the haemopoietin receptor superfamily has been implicated in mediating dimerization during receptor activation. Specific residues in the haemopoietin homology domain (residing within or adjacent to the loop between the a′ and b′ β-strands) of GH receptor monomers participate in hydrogen bonding and salt bridges to help stabilize ligand-induced receptor homodimers (Figure 1 and de Vos *et al.*, 1992).

Table I. Establishment of factor-independent cell clones and analysis of proviral integration

Cells	Frequency of expansion of agar colonies in liquid culture[a]	No. of proviral integrations per clone[b]
FDC-P1-*mpl* R117C	13/13	4.3 ± 2.5 (*n* = 6)
FDC-P1-*mpl* S368C	12/12	1.6 ± 0.5 (*n* = 9)
FDC-P1-*mpl* S369C	15/16	3.2 ± 2.1 (*n* = 6)
Ba/F3-*mpl* R117C	0/1	–
Ba/F3-*mpl* S368C	12/12	4.2 ± 1.5 (*n* = 6)
Ba/F3-*mpl* S369C	12/12	7.8 ± 3.1 (*n* = 6)

[a]Colonies growing in the absence of exogenous growth factors in primary agar cultures of infected cells were picked into DMEM + 10% FCS. The number of colonies that expanded into an established line is expressed as a ratio of the number of colonies transferred.
[b]Mean ± standard error of the number of independent proviral integration sites assessed using both c-*mpl* cDNA and *neo* probes in Southern blot analysis of established clones of factor-independent cells.
n denotes the number of clones analysed.

Fig. 3. (A) Efficiency of induction of factor independence by *mpl* mutants. Colony formation in agar by uninfected Ba/F3, Ba/F3-LXSN and Ba/F3 cells expressing wild-type (wt) or mutant (R117C, S120C, S368C, S369C) Mpl receptors in response to WEHI3B-D⁻-conditioned medium (W3BCM), TPO or in the absence of exogenous factors (no factor) is shown. Cells expressing Mpl receptors were pre-selected for 14 days in liquid cultures with TPO. Ba/F3-LXSN cells were selected in W3BCM plus G418. The mean and standard error of triplicate cultures are graphed. 48% of the number of Ba/F3-*mpl*S368C cells and 7% of Ba/F3-*mpl*S369C cells that grew in TPO were autonomous. No autonomous cells were detected in any of the other cell populations. (B) Dose–response of infected Ba/F3 cell populations to TPO. Two-hundred uninfected Ba/F3 cells (●) or Ba/F3 cells expressing wild-type (▲), R117C (△), S120C (□), S368C (○) or S369C (■) *mpl* mutants were placed in microwells with serially diluted concentrations of TPO. The mean of duplicate cell counts after 48 h is shown. Infected cell populations were derived as in (A). The Ba/F3-*mpl*S368C and Ba/F3-*mpl*S369C populations contain factor-independent cells and are therefore maximally stimulated at all TPO concentrations.

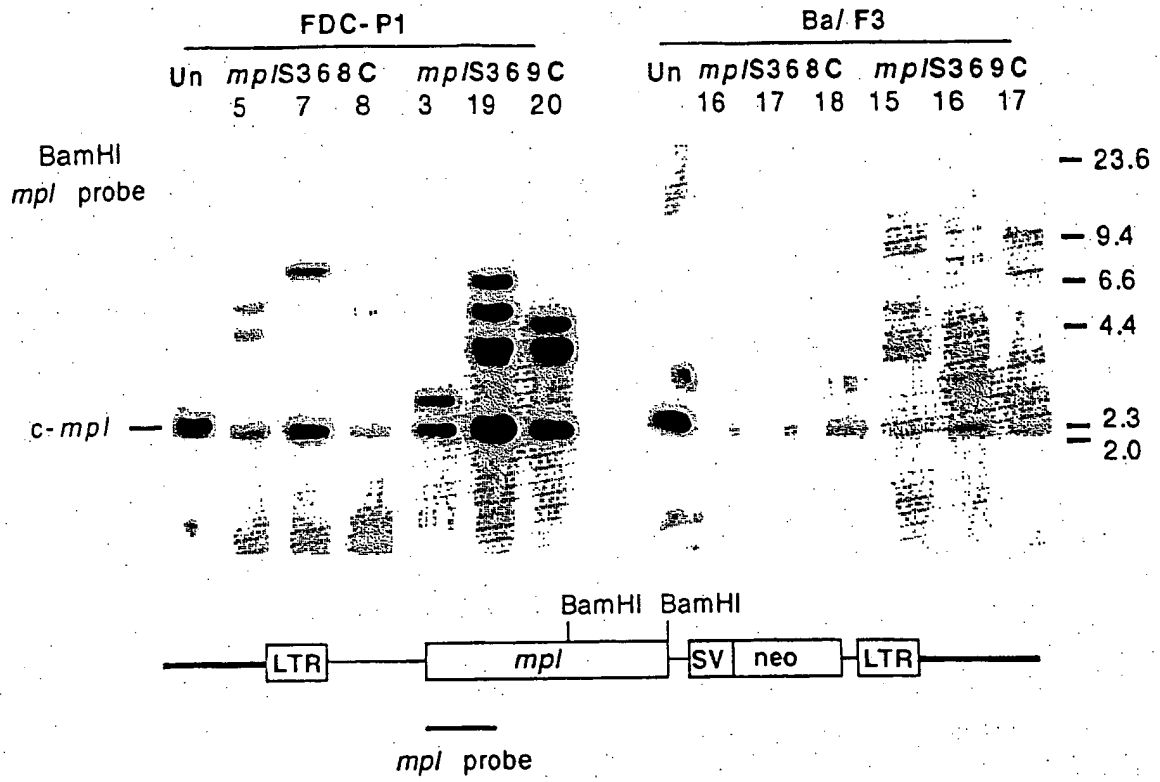


Fig. 4. Proviral integration in factor-independent cell clones. Southern blots of genomic DNA from uninfected (Un) and individual numbered clones of established FDC-P1- and Ba/F3-*mpl*S368C and *mpl*S369C cells. The DNA was digested with *Bam*HI, which cuts within the *mpl* proviruses such that individual proviral integrants will yield uniquely sized fragments with the *mpl* probe indicated. The position of migration of the endogenous c-*mpl* allele and molecular weight size markers in kbp are also shown.

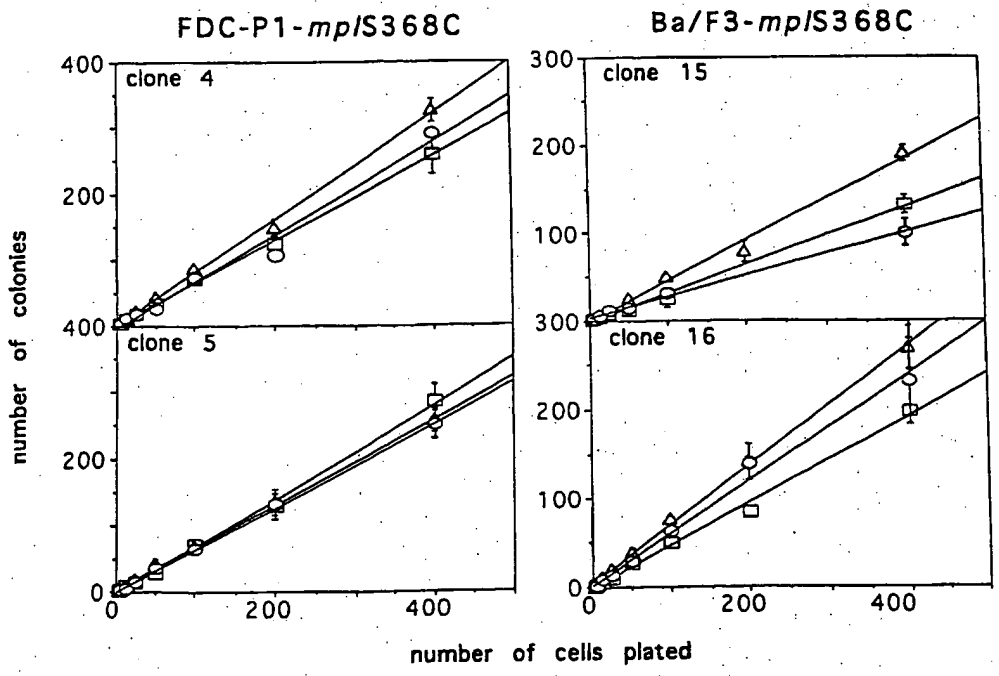


Fig. 5. Density-independent proliferation of factor-independent cells. Individual clones of FDC-P1- and Ba/F3-*mpl*S368C cells were plated at various densities in agar cultures with saturating doses of WEHI3B-D⁻-conditioned medium (△), TPO (○) or in the absence of added factors (□). The mean and standard error of triplicate determinations is shown.

The analogous region of the EPO receptor also seems to mediate dimerization, as the substitution of cysteine residues for particular amino acids in this domain results in ligand-independent receptor activation through disulfide-bonded homodimerization (Yoshimura *et al.*, 1990; Watowich *et al.*, 1992, 1994). The results presented here demonstrate that the c-Mpl receptor also can be activated constitutively by the introduction of cysteine residues into

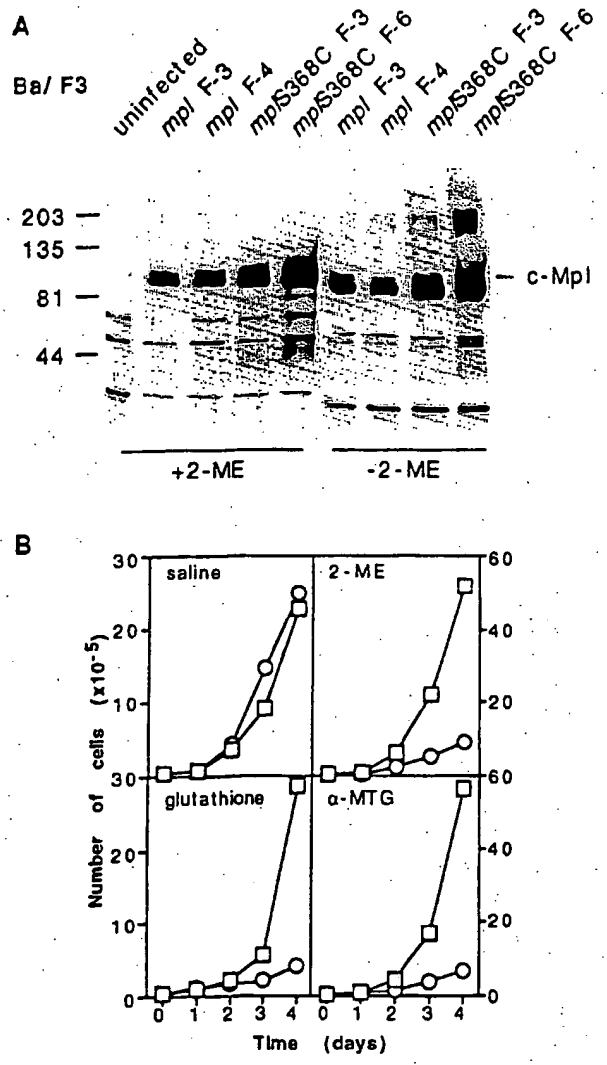


Fig. 6. (A) c-Mpl protein in Ba/F3 cells expressing FLAG epitope-tagged wt (*mpl* F-3, F-4) and S368C mutant (*mpl*S368C F-3, F-6) receptors. Proteins precipitated with the anti-FLAG M2 antibody were left untreated or incubated with 2% 2-ME, separated by SDS–PAGE and blotted to nitrocellulose membranes. The Western blot using the same M2 antibody is shown. The predicted size of the c-Mpl protein (monomeric) is indicated, as are molecular weight standards in kDa. (B) Inhibition of factor-independent cell proliferation by chemical reducing agents. FDC-P1-*mpl*S368C cells were plated in liquid cultures containing 2-ME (1.6×10^{-4} M), glutathione (reduced form; 1.2×10^{-2} M), α-MTG (1.0×10^{-2} M) or in control cultures (saline). Proliferation in the presence (□) or absence (○) of TPO was monitored daily by counting viable cells in each culture.

homologous dimer interface regions identified within its haemopoietin domains. Factor-dependent FDC-P1 or Ba/F3 cells expressing these activated mutant Mpl receptors no longer required exogenous factors for their survival and growth (Figure 2). Their autonomous proliferation was not dependent on cell density (Figure 5) and no secreted factors that stimulate cell growth could be detected, consistent with a constitutive proliferative signal emanating directly from the mutant receptor. Biochemical studies in cells expressing the *mpl*S368C mutant revealed a Mpl protein species of dimeric size that reduced to monomers upon treatment with 2-ME. Moreover, reducing agents inhibited the factor-independent growth of FDC-P1-*mpl*S368C cells (Figure 6). Together, these data strongly support the model that the introduction of cysteine residues into a dimer interface homology domain of c-Mpl induces constitutive receptor activity through disulfide-bonded homodimerization.

Previous studies with G-CSF receptor–c-Mpl chimeras (Vigon *et al.*, 1993b; Baumann *et al.*, 1994) and a variant of the constitutively active v-Mpl oncoprotein (Courtois *et al.*, 1995) have demonstrated that homodimerization of the Mpl intracellular domain induces receptor activity. Our studies here confirm these observations and extend them by providing strong evidence that receptor homodimerization is involved in the normal process of ligand-mediated Mpl activation. Our results suggest that the GH receptor dimer interface domain is conserved functionally in the loop region between the a′ and b′ β-strands of the haemopoietin domain, not only in the EPO receptor but also within c-Mpl. Given the structural similarity of haemopoietin receptor family members, the analogous regions of other haemopoietin receptors may also mediate the subunit associations that characterize their activation. Indeed, in mutation studies of the G-CSF receptor, the N-terminal half of the haemopoietin domain is sufficient to bind G-CSF at low affinity, but for high affinity binding, which correlates with homodimer formation (Fukunaga *et al.*, 1990), the full domain must be present (Fukunaga *et al.*, 1991). Several IL-6 receptor α-chain mutants have been described that can bind IL-6 but are unable subsequently to interact with gp130 for signal transduction. However, in this receptor, most of the mutations cluster in the region of the predicted e′ and f′ β-strands of the haemopoietin domain (Yawata *et al.*, 1993). Intriguingly, although the majority of residues contributing to stabilization of GH receptor dimers reside in the a′–b′ loop region, two are also located in the e′ β-strand, which lies adjacent (de Vos *et al.*, 1992). If the mechanism of subunit interaction is indeed similar among the haemopoietin receptors, a conserved subunit interface may thus be composed of two domains, although their relative contribution may vary between receptors.

The emergence of key domains in haemopoietin receptor subunit interactions has important implications for receptor function. Two independent cases of Laron syndrome, a familial resistance to GH, have arisen through mutations in the dimer interface domain of the GH receptor that prevent ligand-induced homodimerization (Duquesnoy *et al.*, 1994). Thus, in addition to being potential sites for mutational activation, dimer interface domains such as that identified here in c-Mpl, may also provide a target for approaches designed to inhibit haemopoietin receptor function. Our studies also highlight parallels between activation of haemopoietin receptors and receptors of the structurally unrelated tyrosine kinase family. Dimer formation is associated with activation of many tyrosine kinase receptors, and mutations that stabilize dimerization have been found to stimulate kinase activity and transforming capacity (Weiner *et al.*, 1989; Sorokin *et al.*, 1994).

c-Mpl belongs to the minority of haemopoietin receptors that have two haemopoietin homology domains within their extracellular sequence. Substitution of cysteine residues into either of these domains yielded receptors with constitutive activity. However, of the two receptors derived by mutations within the N-terminal domain, only MplR117C displayed very weak TPO-independent activity.

Table II. Tumorigenicity of factor-independent *mpl*S368C cells

Cells injected[a]	Mouse strain (irradiation dose)	Mice developing tumours/No. injected	Tumor latency (days)
FDC-P1	DBA/2	0/3	
FDC-P1-*mpl*S368C.4	DBA/2	3/3	20, 20, 26
FDC-P1-*mpl*S368C.5	DBA/2	3/3	16, 17, 27
FDC-P1-*mpl*S368C.12	DBA/2	3/3	28, 22, 23
Ba/F3	Balb/c	0/3	
	Balb/c (500 rad)	0/3	
Ba/F3-*mpl*wt	Balb/c (500 rad)	0/3	
Ba/F3-*mpl*S368C.15	Balb/c	0/3	
	Balb/c (500 rad)	2/3	34, 34
Ba/F3-*mpl*S368C.16	Balb/c	0/3	
	Balb/c (500 rad)	1/3	40
Ba/F3-*mpl*S368C.18	Balb/c	0/3	
	Balb/c (500 rad)	1/3	70

[a]10^6 cells were injected subcutaneously into the flank of each mouse.

This was not due to poor expression, deficiencies in transport to the cell surface or defective signal transduction, as cells expressing MplR117C or MplS120C could respond to TPO as efficiently as cells expressing Mplwt (Figure 3). In contrast, both membrane-proximal domain mutants (MplS368C and S369C) displayed constitutive activity (Figures 2 and 3). With the caveat that the precise residues within the N-terminal domain may not have been targeted for optimal activation in our study, these results may indicate a more significant contribution to Mpl activation by the membrane-proximal dimer interface homology domain. Even within the membrane-proximal domain, the two independent Mpl mutants appeared to differ in potency. Of all Ba/F3 cells expressing sufficient levels of the *mpl*S368C mutant to respond to TPO, around half were autonomous. In contrast, only 7% of TPO-responsive Ba/F3-*mpl*S369C cells were factor independent (Figure 3A). Similarly, only a subset of residues in the EPO receptor dimer interface domain induce constitutive activity when substituted with cysteines (Watowich *et al.*, 1992). In the homodimerization model, it seems likely that, at different positions within the dimer interface domain, the orientation of substituted cysteine residues will influence critically the efficiency of disulfide bond formation between adjacent mutant receptors. Higher efficiency of bond formation between *mpl*S368C monomers than those of *mpl*S369C could explain their differential capacities to induce factor-independent colony formation. If a threshold level of homodimer formation is required to stimulate cellular proliferation, higher numbers of receptors may be required in autonomous *mpl*S369C cells than in equivalent *mpl*S368C cells. We have observed a comparatively higher average number of *mpl*S369C retroviral integration sites (Figure 4 and Table I), which may indicate selection for elevated expression levels.

In addition to becoming factor independent, FDC-P1 and Ba/F3 cells expressing the *mpl*S368C mutant also induced tumours in transplanted mice. FDC-P1-*mpl*S368C cells were highly tumorigenic, with all injected mice from three independent clones succumbing within 2–4 weeks. Ba/F3-*mpl*S368C cells were less potent and also required prior irradiation of the recipient mice (Table II). Although v-*mpl*, albeit a grossly mutated receptor form, is highly tumorigenic (Wendling *et al.*, 1986), to date the cellular *mpl* gene has not been found to be reproducibly over-expressed in human myeloid leukaemias (Vigon *et al.*, 1993a). Our results extend the studies of v-*mpl* to show that deletion of extracellular receptor domains and/or the presence of viral *env* sequences are not mandatory for constitutive receptor activity, and further demonstrate that, when activated by point mutation, the cellular *mpl* gene is tumorigenic in established haemopoietic cell lines. It is feasible, therefore, that subtle alterations to the c-*mpl* gene which influence receptor activity may be found to contribute to the development of leukaemia.

Materials and methods

In vitro mutagenesis

Mutations were engineered into the murine *mpl* cDNA using a modification of the site-directed mutagenesis technique of Kunkel (1985). Briefly, a phagemid containing the full-length c-*mpl2* cDNA (Alexander and Dunn, 1995) in BluescriptKS(+) was transformed into *Escherichia coli* CJ236 (*dut*$^-$, *ung*$^-$) cells from which single-stranded, dUTP-containing template DNA was prepared. Each mutagenesis oligonucleotide (250 ng) was annealed to the template DNA (500 ng) and then incubated with T7 DNA polymerase (2 U; Sequenase, USB, Cleveland, OH) and T4 DNA ligase (1 U; Promega, Madison, WI) in Sequenase buffer supplemented with 650 μM dNTP, 500 μM ATP and 1.5 mM dithiothreitol (DTT) to synthesize and ligate the mutated phagemid strand. This reaction product was transformed into *E.coli* NM522 (*dut*$^+$, *ung*$^+$) cells to select against the dUTP$^+$ parental strand. Individual clones were initially screened for the presence of novel restriction endonuclease sites (see below), and then sequenced to confirm the introduction of the desired mutation and to exclude secondary mutations. The mutagenesis oligonucleotides, designed to alter specific codons in c-*mpl* to those for cysteine, and also incorporate silent nucleotide changes to introduce novel restriction endonuclease sites (*Stu*I for *mpl*R117C, *Sma*I for *mpl*S120C, *Pst*I for *mpl*S368C and *Aat*II for *mpl*S369C), are antisense to the c-*mpl* coding strand and have the following sequences: *mpl*R117C 5′-GGCTCCCACCGCAGGCCTTGATG-3′; *mpl*S120C 5′-GAAGTTC-CCCGGGTTGGCACCCACCCCTG-3′; *mpl*S368C 5′-CTCCAGCC-TTCCACTGCAGACCTCCCTCCAGTG-3′; *mpl*S369C 5′-CCACTC-CAACTCCAGACGTCCGCATGAGACCTCCCTCCA-3′. The codon numbers 117, 120, 368 and 369 represent amino acid positions in the mature Mpl protein (Skoda *et al.*, 1993), while underlined nucleotides indicate residues altered from the wild-type sequence. Each *mpl* mutant, as well as the wild-type cDNA, was incorporated into the LXSN retroviral vector (Miller and Rosman, 1989) in which the receptor is expressed from the 5′ long terminal repeat. The vector also includes an internal *neo*^{R} gene driven by an SV40 promoter allowing selection for infected cells with geneticin (G418 sulfate, Gibco, NY). Stable cell lines releasing infectious, helper-free virus were obtained from these receptor constructs via transfection into Ψ-2 packaging cells (Mann *et al.*, 1983) and selection for G418 resistance.

Cell lines and retroviral infections

The FDC-P1 (Dexter *et al.*, 1980) and Ba/F3 (Palacios and Steinmetz, 1985) haemopoietic cell lines are strictly dependent on exogenous factors, including IL-3, for survival and growth and were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 10% WEHI-3BD$^-$-conditioned medium (Metcalf, 1984). Cells were infected with retroviruses expressing wild-type or mutant Mpl receptors by exposure to viral supernatants for 2 h or by co-cultivation with Ψ-2 virus-producing cells for 48 h in growth medium supplemented with 4 μg/ml polybrene (hexadimethrine bromide, Sigma, St Louis, MO).

Agar cultures

Infected cells or factor-independent clones were washed by centrifugation in phosphate-buffered saline (PBS), resuspended at the desired concentration in DMEM containing 10% FCS and 0.3% agar (Metcalf, 1984) and plated as 1 ml cultures in 35 mm Petri dishes. Cultures were stimulated with WEHI-3BD$^-$-conditioned medium (10%, as a source of IL-3), the medium conditioned by COS cells expressing recombinant human TPO, kindly provided by Drs T.Willson and M.Rossner (The Walter and Eliza Hall Institute of Medical Research), or with normal saline. Where added, G418 was used at 1.2 mg/ml. Colony formation was scored after 7 days incubation at 37°C in a fully humidified atmosphere of 10% CO_2 in air.

Microwell assays

The response of cell lines to TPO was measured in Lux 60 microwell HLA plates (Nunc Inc., Roskilde, Denmark). Cells were washed in DMEM containing 10% FCS and 10 μl aliquots containing 200 cells in the same medium were placed in each microwell with 5 μl of serially diluted conditioned medium containing TPO. The numbers of viable cells in each well were counted after incubation for 2 days at 37°C in a fully humidified atmosphere of 10% CO_2 in air. Serially diluted media conditioned by factor-independent clones were assayed in identical fashion for growth stimulatory activity on Ba/F3 cells engineered to exogenously express the normal Mpl receptor.

Nucleic acid analyses

For genomic DNA extraction, cells were washed twice in PBS and resuspended in 50 mM Tris–HCl, 100 mM EDTA, 100 mM NaCl, 1% SDS, 500 μg/ml proteinase K at pH 8. After incubation at 50°C for 16 h, the lysate was extracted twice with a 25:25:1 mixture of phenol, chloroform and iso-amyl alcohol (PCIAA) and the genomic DNA precipitated with isopropanol in the presence of 300 mM sodium acetate. For Southern blots, 15 μg of DNA were digested to completion with the desired restriction endonuclease and then electrophoresed through 0.7% agarose gels. The DNA was transferred to nylon membrane (Genescreen Plus, NEN, Boston) by soaking the gel sequentially in 0.25 M HCl and then 0.5 M NaOH, 1.5 M NaCl for 20 min each before blotting with the same alkali buffer. After transfer, the membranes were neutralized in 40 mM Na_2HPO_4, pH 7.2.

RNA was purified by dissolving cells in 4 M guanidine thiocyanate, 25 mM sodium citrate, 1% 2-ME, 0.5% lauryl sarcosine, then extracting the lysate with PCIAA and precipitating the RNA from the aqueous phase with isopropanol. The polyadenylated RNA fraction was purified by oligo(dT) chromatography. Briefly, total cellular RNA was dissolved in 0.5 M NaCl, 10 mM EDTA, 20 mM Tris–HCl, pH 7.5 and incubated with 50–100 mg oligo(dT)-cellulose (Boehringer, Mannheim) at room temperature for 1–2 h. The cellulose was washed thoroughly in 0.4 M NaCl, 10 mM EDTA, 0.2% SDS, 20 mM Tris–HCl, pH 7.5 and then 0.1 M NaCl, 10 mM EDTA, 0.2% SDS, 10 mM Tris–HCl, pH 7.5 before the poly(A)$^+$ RNA was eluted in 1 mM EDTA, 0.2% SDS, 1 mM Tris–HCl, pH 7.5 at 60°C. Northern blots were performed by electrophoresis of poly(A)$^+$ RNA (5 μg) through 1.2% agarose gels containing 6% formaldehyde followed by blotting to nylon membranes (Genescreen Plus, NEN, Boston) in 10× SSC (1.5 M NaCl, 150 mM sodium citrate, pH 7.0).

Northern and Southern blot membranes were hybridized with cDNA probes labelled with [α-^{32}P]dATP using a random decanucleotide priming system (Bresatec, Adelaide, South Australia) and separated from unincorporated radioactivity by ethanol precipitation from 2.5 M ammonium acetate. After hybridization in 0.5 M Na_2HPO_4, 7% SDS, 1 mM EDTA, pH 7.2 at 65°C for 16 h, membranes were washed twice in 40 mM Na_2HPO_4, 1% SDS, pH 7.2 for 30 min at 65°C and exposed to autoradiographic film. The *mpl* probe used was a 0.23 kb fragment of c-*mpl* cDNA extending from the initiation ATG codon to the *Sph*I site downstream.

Protein analyses

Ba/F3 cells (10^6) expressing normal or mutant Mpl receptors engineered with a FLAG (Asp–Tyr–Lys–Asp–Asp–Asp–Asp–Lys) peptide epitope at the N-terminus of the mature protein were lysed in 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 50 mM Tris–HCl, pH 7.4, for 5 min on ice in the presence of protease inhibitors. Lysates were clarified by centrifugation and Mpl receptors precipitated with anti-FLAG M2 affinity gel (Eastman Kodak, New Haven, CT) for 2 h at 4°C. Immunoprecipitates were washed twice in lysis buffer, half of each sample was incubated for 5 min with 2-ME (2%) and, after boiling in 1% SDS, 10% glycerol, 80 mM Tris–HCl, pH 6.8, were separated by electrophoresis in SDS–polyacrylamide gels and electrotransferred to nitrocellulose membranes (Amersham, UK). Filters were incubated with the same M2 antibody and protein visualized using the Amersham (UK) ECL system.

Transplantation

Factor-independent FDC-P1 and Ba/F3 clones were assayed for tumorigenicity in 8- to 20-week-old syngeneic DBA-2 and Balb/c mice respectively. Mice receiving Ba/F3 cell clones were divided into two groups, one of which was irradiated with 500 rad whole body irradiation prior to injection. 10^6 cells were washed twice in DMEM and injected subcutaneously into the flank region of each of three mice per assay.

Acknowledgements

We thank Maria Harrison-Smith for excellent technical assistance, Dr D.Hilton for assistance with amino acid alignments, Dr M.Hibbs for advice on *in vitro* mutagenesis, Drs M.Rossner and T.Willson for providing TPO and Dr N.Nicola and Professor A.Burgess for comments on the manuscript. This work was supported in part by an Australian National Health and Medical Research Council C.J.Martin Fellowship to W.S.A.

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Received on June 16, 1995; revised on August 18, 1995

Structures of the extracellular domain of the type I tumor necrosis factor receptor

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Background: Tumor necrosis factor (TNF) is a powerful cytokine that is involved in immune and pro-inflammatory responses. Two TNF receptors that belong to the cysteine-rich low affinity nerve growth factor receptor family (TNF-R1 and TNF-R2) are the sole mediators of TNF signalling. Signalling is thought to occur when a trimer of TNF binds to the extracellular domains of two or three receptor molecules, which permits aggregation and activation of the cytoplasmic domains. The complex is then internalized within an endocytic vesicle, whereupon it dissociates at low pH. Structure of the soluble extracellular domain of the receptor (sTNF-R1) both in the unliganded and TNF-bound state have previously been determined. In both instances, the fourth subdomain of the receptor was found to be partly disordered. In the unliganded state at pH 7.5, the extracellular domain forms two distinct types of dimer, parallel and antiparallel; the antiparallel dimer occludes the TNF-binding.

Results: We have determined the structure of sTNF-R1 in two crystal forms in high salt at pH 3.7. The orthorhombic crystals diffract to 1.85 Å and the entire polypeptide is well ordered. In contrast, the C-terminal 32 residues are disordered in the hexagonal crystals. In the orthorhombic form, these residues exhibit a topology and disulphide connectivity that differs from the other three cysteine-rich domains in the molecule. In both forms, the interface is considerably more extensive than that used in complex formation with LTα. This 'low pH' dimer is different from both of the dimers observed in crystals grown at pH 7.5.

Conclusions: The occurrence of the antiparallel dimers in both low pH crystal forms suggest that they are not an artefact of crystal packing. Such dimers may form in the low pH environment of the endosome. Because the dimer contact surface occludes the TNF-binding site, formation of this dimer would dissociate the TNF–receptor complex within the endosome. Three of the four cysteine-rich domains of TNF-R1 are constructed from two distinct structural modules, termed A1 and B2. The fourth subdomain comprises an A1 module followed by an unusual C2 module. Although the orientation of these modules with respect to each other is sensitive to crystal packing, ligand binding, pH and ionic strength, the modules are structurally well conserved between and within the known sTNF-R1 structures.

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Key words: crystal structures, molecular replacement, receptor, signalling, TNF

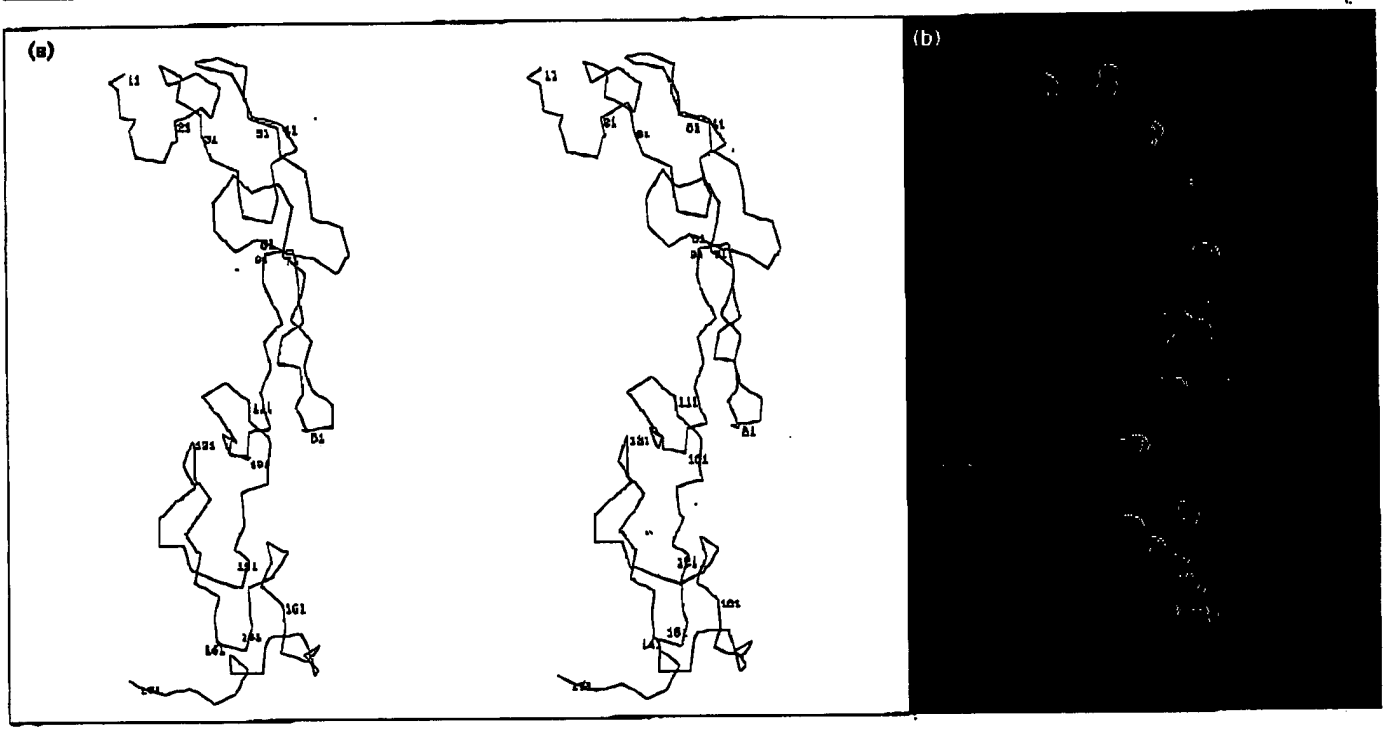
Received: 8 July 1996
Revisions requested: 27 July 1996
Revisions received: 20 August 1996
Accepted: 6 September 1996

Structure 15 November 1996, 4:1251–1262

Introduction

Tumor necrosis factor α (TNFα) and the closely related protein lymphotoxin (LTα/TNFβ) are now recognized as two of the most pleiotropic cytokines, signalling a large number of cellular processes, including cytotoxicity, apoptosis, antiviral activity and immunoregulatory activity [1]. The effects of TNF (denotes both cytokines) are signalled through two receptors: type I (55 kDa) and type II (75 kDa) [2–4]. The receptors belong to the cysteine-rich low affinity nerve growth factor receptor superfamily (NGF-R) which includes FAS, CD40, OX40, CD27 receptor and several viral proteins [5]. Across the family there is little primary sequence similarity among the extracellular domains, with the exception of the pattern of cysteines, and none at all within the intracellular domain. The receptors are structurally distinct from the cysteine knot class of proteins such as human chorionic gonadotropin (HCG) [6]. The X-ray structures of sTNF-R1 (the soluble extracellular domain of TNF-R1), both unliganded [7] and its complex with LTα [8], shows the receptor to be an elongated molecule, with a ladder-like progression of disulfide bonds along the long axis of the molecule. A linear combination of four subdomains was identified in the structure. The C terminus of the fourth subdomain is disordered in both structures and its primary sequence is inconsistent with the subdomain structure of the other three.

Figure 1



The complete structure of the monomer of sTNF-R1. (a) A stereo diagram of the numbered Cα trace of the sTNF-R1 molecule found at pH 3.7. (b) A ribbon representation of the structure with disulphide bonds shown as yellow spheres, prepared with RASTER3D [31]. Subdomains one and three are in red and subdomains two and four in orange.

Signal transduction occurs when TNF binds to the extracellular domains of two or three receptor molecules. This event promotes aggregation [9,10] of the intracellular domains, which in turn stimulates their interaction with a variety of other signalling molecules [11,12]. After signalling, the complex is internalized, and dissociates in the low-pH environment of the endosome [13]. The extracellular domain of the receptor is found as a soluble protein (sTNF-R1) in serum and may act to downregulate TNF signalling [14]. In the 2.85 Å crystal structure of LTα complexed to sTNF-R1 [8], three sTNF-R1 molecules are bound parallel to the LTα trimer axis. One sTNF-R1 molecule is bound at each of the three LTα–LTα interfaces. Interactions with LTα are confined to two clusters, essentially the C-terminal half of the second subdomain (residues 56–74) and much of the third subdomain (residues 77–81 and 107–114). The receptor extracellular domains do not contact each other in the complex.

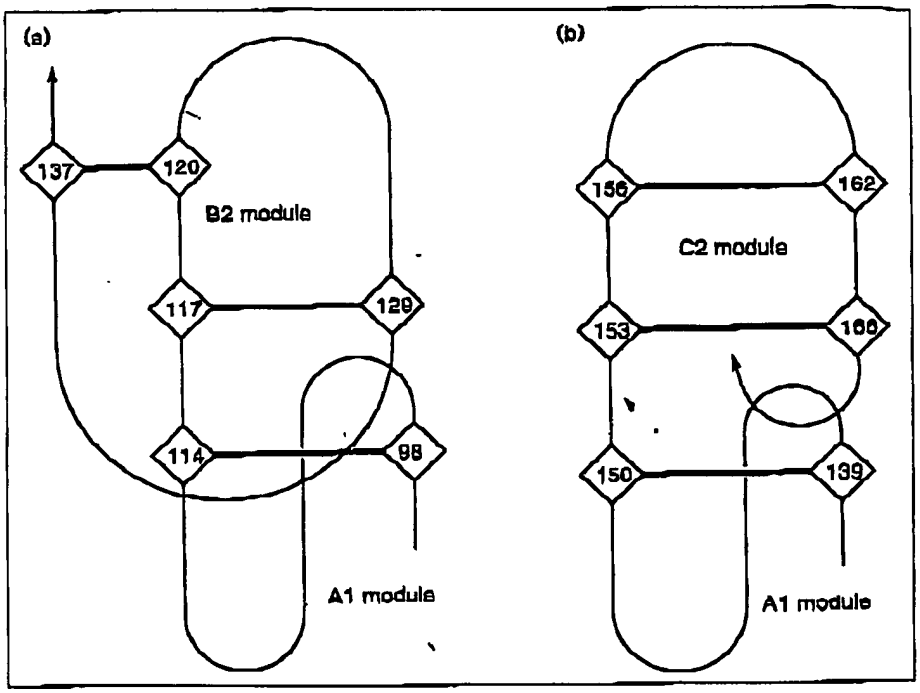
The X-ray structure of unliganded sTNF-R1, crystallized from 2-methylpentan-2,4-diol (MPD) at pH 7.5 [7], raised the possibility that TNF release induces quaternary structural changes in the extracellular domain that could regulate the activity of the receptor. This structure showed that sTNF-R1 in the absence of TNF forms two distinct types of dimer one of which occludes the TNF- binding site [7]. A regulatory function for the ligand- binding domain is suggested by the aggregation and signalling activity shown by intracellular domains expressed in the cytosol in the absence of TNF [15]. In contrast, intact receptors are tightly regulated and silent in the absence of TNF. One interpretation of these results is that, in some way, the extracellular domains prevent 'false' signalling in the absence of TNF.

We suggested that dimerization of the extracellular domain could prevent signalling. We observed that one of the dimers, in which the extracellular domains are arranged in a parallel fashion, did not occlude the TNF-binding site. Such dimers could promote the formation of large cell surface aggregates that could play a role in complex internalization or may concentrate the signal within the cell. However, such aggregates were not observed in the crystalline LTα–sTNF-R1 complex [8].

We report two new structures of unliganded sTNF-R1, obtained at high ionic strength and pH 3.7. Both crystal forms diffract X-rays to d spacings less than 1.9 Å. In one form, the entire polypeptide chain (corresponding to residues 11–172 of full-length human TNF-R1) is ordered.

Figure 2

A disulphide connectivity diagram of (a) the third and (b) fourth subdomains. The disulphide connectivity is clearly different in the fourth subdomain.



The C terminus of the fourth subdomain of sTNF-R1 has a different disulfide connectivity from that previously proposed [8] and a fold that is different from the C termini of the other three subdomains. At low pH the receptor is found as a dimer with extensive interactions between monomers, which occludes the TNF interaction surface. We suggest that this dimer may form during endocytosis, disrupting the TNF–TNF-R1 complex and allowing recycling of the receptor [16]. Consistent with an observation by Bazan [5], we find that each subdomain comprises two distinct types of structural modules. The modules, rather than the subdomains, are structurally conserved within the sTNF-R1 molecule.

Results and discussion

The monomer and the low pH dimer

From a concentrated solution of magnesium sulphate buffered at pH 3.7, crystals of sTNF-R1 appear simultaneously in hexagonal and orthorhombic space groups. The sTNF-R1 monomer (Fig. 1) found in the orthorhombic space group is highly elongated, with a solvent accessible surface area [17] of approximately 12000 Å^2 (0.48:0.52, apolar:polar) and an accessible surface to volume ratio of 0.72 Å^{-1}, seven times greater than for a sphere of equivalent volume. The monomer has no hydrophobic core but buries over 90% of the solvent-accessible area of the sulphur atoms in the disulphide bonds. The overall fold of the protein has been described [7,8]. The C terminus of the fourth subdomain (153–172), which was disordered in structures previously reported [7,8], is ordered in the orthorhombic crystals, but not in the hexagonal form. The disulphide pattern Cys3–Cys6 and Cys4–Cys5, is also different from the other subdomains (Fig. 2) which leads to a structure with a topology different from that observed in the other three subdomains (Figs 1,2). With the crystal structures described here, the structure of sTNF-R1 has been observed in six distinct packing environments. Within

Table 1

Interactions that constitute the dimers and LTα complex of sTNF-R1.

Interaction	pH 3.7 dimer	pH 7.5 antiparallel dimer	pH 7.5 parallel dimer	LTα complex
Hydrogen bonds*	12	11	11	14
van der Waals†	112	108	149	115
Ion pairs‡	1	2	1	4
Bridging waters	15	5	10	1
Contact surface§				
polar(Å^2)	1402	878	1084	1014
apolar(Å^2)	1480	1597	1079	1154
total(Å^2)	2882	1475	2149	2168
Complementarity#	0.65	0.70	0.67	0.66

*Contacts closer than 3.3 Å with appropriate stereochemistry; †contacts closer than 4.0 Å; ‡contacts closer than 3.9 Å involving two oppositely charged groups (at pH 3.7 we do not expect these to be charged interactions); §defined using a 1.4 Å probe [17]; #calculated by the method of Lawrence and Coleman [33].

Table 2

Non-bonded contacts between antiparallel dimers in orthorhombic (pH 3.7) crystals of sTNF-R1.

	Polar contacts*				van der Waals
Residue A	Atom A	Residue B	Atom B	Distance (Å)	(≤4.0 Å)†
D12	–	–	–	–	N155
V14	–	–	–	–	R146, V151, L160(3)
C15	O	R146	Nη1	3.2	R146(2), L160(2)
P16	–	–	–	–	S159(2), L160, E161(2)
Q17	N	E161	Oε2	3.3	–
I21	–	–	–	–	E147(2)
F60	–	–	–	–	L111
H89	–	–	–	–	E79(4)
L71	–	–	–	–	R77, M80(2), L111
S72	O	R77	Nη1	3.2	R77(3)
	O	R77	Nη2	3.1	–
S74	–	–	–	–	R77
R77	Nη1	S72	O	3.1	L71(2), S72(3), D93
	Nη2	S72	O	3.2	–
	Nη2	S74	Oγ	3.3	–
E79	–	–	–	–	L67, L71
D93	–	–	–	–	L111
R104	Nη1	E109	Oε2	3.0	E109(2)
Y106	Oη	E109	Oε1	2.8	E109(4)
W107					S93(3)
N110	Oδ1	N110	Nδ2	2.7	N110(6)
L111	–	–	–	–	F60
F144	–	–	–	–	V14(2)
R146	Nη1	C15	O	3.0	V14(3), C15(4), P16, Q17(2)
E147	Oε1	C15	N	2.9	M11(2), V14(5), C15(4)
V151	–	–	–	–	M11(2), V14
N155	–	–	–	–	D12(14)
S159	–	–	–	–	P16(2)
L160	–	–	–	–	V14
E161	Oε1	Q17	N	2.8	P16(6), Q17(3)

*Hydrogen bond and ion pairs between atoms from monomer A and atoms from monomer B (cut-off is 3.3 Å). Residues are identified by the one letter code and sequence number. †For each residue in monomer A, van der Waals partners in monomer B are listed. The number in parentheses indicates the number of atoms in that residue within 4.0 Å of the corresponding residue in monomer A.

this group, the conformation of the C-terminal subdomain ranges from completely disordered to fully ordered.

In both crystal forms, the prominent structural unit is a dimer of sTNF-R1 molecules (Fig. 3). However, the packing arrangements by which the dimer is organized within the hexagonal and orthorhombic cells differ. The conservation of this dimer in the context of two different crystal-packing arrangements suggests that dimer formation is driven by solvent environment and pH rather than crystal-packing forces. The sTNF-R1 dimer found in the orthorhombic and hexagonal crystal forms is different from both of the dimers we previously observed in the tetragonal crystal form grown from MPD at pH 7.5 (Fig. 4) [7]. Although these differences in the dimer association between the tetragonal and orthorhombic/hexagonal forms may be attributed to either pH and/or the solvent conditions, we refer to the dimers either as 'high pH' (tetragonal) and 'low pH' (orthorhombic, hexagonal). Like one of the two high pH dimeric species, the low pH dimer is formed by an antiparallel (head-to-tail) arrangement of the monomers. Unlike the antiparallel high pH dimer (Fig. 4b), the two monomers completely overlap, such that the length of the dimer is 90 Å, almost identical to that of a single monomer. There appears to be no obvious explanation for why one dimer is favoured under different solvent conditions. The low pH dimer remains extremely non-globular with an accessible surface to volume ratio of 0.64 $Å^{-1}$ (nearly six times greater than for a sphere of equivalent volume). In the orthorhombic form, a noncrystallographic twofold axis relates the monomers. The N terminus of monomer 2 interacts extensively with the C terminus of monomer 1, presumably ordering the two termini. However, the opposite pair of termini do not form such close contacts with each other, leading to disorder in residues 11 and 12 in monomer 1 and residues 168–171 in monomer 2. In the hexagonal crystal form an essentially identical dimer is generated by a crystallographic twofold axis. However, the C-terminal 32 residues are disordered in this structure.

Figure 3

Two orthogonal views of the low pH dimer that is the asymmetric unit of the $P2_12_12_1$ crystal form. Careful examination of the figure shows that the interaction between subdomain one and subdomain four is deeper at one end of the dimer than the other. (Figure prepared using RASTER3D [31].) The color scheme for one monomer is the same as in Figure 1. The other monomer is colored dark blue (subdomains one and three) and bright blue (subdomains two and four).

The low pH dimer is held together by extensive hydrophobic and hydrophilic interactions that bury 1480 Å^2 of hydrophobic accessible surface and 1400 Å^2 of hydrophilic accessible surface These interactions, which are detailed in Tables 1 and 2, occur along the entire length of the dimer. Two cavities (related by the twofold axis) are formed at the dimer interface with residues 17, 18, 32 and 67 from one monomer and residues 113, 115, 134, 148 from the other. The cavity has an approximate diameter of 10 Å, and is partially filled with ordered water molecules. In addition, the twofold axis of the dimer passes through a narrow twisted solvent channel with a diameter no greater than 3.0 Å. Unlike the recombinant protein described here, the extracellular domain of the native receptor is glycosylated at Asn17, 96 and 124. However, as these sites are not located at the dimer interface they would be expected not to interfere with dimerization.

The surface area buried by the low pH dimer is comparable with that observed in other multimeric proteins [18] and far exceeds that commonly seen for antibody–antigen interactions [19]. Perhaps the most interesting observation is that the low pH dimer buries almost 50% more surface area than the LTα–sTNF-R1 complex [8]. Residues 14–17, 21, 60, 69–72, 77–79, 104–107, 110–114, 144, 146–147, 151–155, 160–161 are involved in or are buried by formation of the low pH dimer (Table 2), encompassing virtually all the residues involved in LTα– recognition [8]. This dimer would therefore be incapable of binding TNF in the manner observed in crystals of the LTα–sTNF-R1 complex [8]. It is notable that the LTα complex is stabilized by four ion pairs; we would expect these to be much weaker at low pH. The low pH dimer bears a further superficial resemblance to the high pH antiparallel dimer: both bury the TNF interaction surface. However, although some residues (His69, Leu71, Ser72, Arg77, Glu79 and Asp93) are present at both interfaces, the dimers are formed by completely different sets of residue–residue contacts (Tables 2 and 3). The parallel dimer found at high pH which does not occlude the TNF interaction surface is held together by an almost completely different subset of residues and contacts than either of the antiparallel dimers (Tables 2, 3 and 4).

As yet no direct evidence exists to support a dimeric receptor species in solution. Nevertheless, in the relevant biological context, TNF-R1 dimers are proposed to form on the two-dimensional surface of the plasma membrane where the entropy penalty for dimerization would be significantly less than for dimer formation in solution [20].

Modular structure of sTNF-R1

The extracellular domains of TNF-R1 and its homologues have been characterized as multiple repeats of an

Table 3

Non-bonded contacts between antiparallel dimers in tetragonal (pH 7.5) crystals of sTNF-R1.

Polar contacts*					van der Waals
Residue A	Atom A	Residue B	Atom B	Distance (Å)	(4.0 Å)†
Q24	–	–	–	–	S108(2)
Y40	O	R77	Nη2	2.7	C76, R77(18), E79(5)
N41	–	–	–	–	L111
R58	Nη2	E79	Oε2	2.9	E79(3)
S59	–	–	–	–	S72
F60	–	–	–	–	H69(3)
H69	Nδ1	S72	O	2.8	F60(3), L71(2), S72(8),S74(2), D83
C70	N	S72	Oγ	2.8	C70, L(2), S72(8)
	O	S72	N	2.9	–
L71	–	–	–	–	H69(3), C70(2)
S72	N	C70	O	2.9	S59, H69(6),C70(7)
	Oγ	C70	N	2.9	–
	O	H69	Nδ1	2.7	–
S74	–	–	–	–	H69(2)
K75	Nζ	E56	Oε1	2.9	Y38, E56
C76	–	–	–	–	Y40
R77	Nη1	Y40	O	3.0	Y40(8), N41(7)
Nη2	Y40	O	2.6	–	–
K78	–	–	–	–	Y40
E79	Oε1	Y40	Oη	2.7	Y40(7)
	Oε2	N41	Nδ2	3.2	–
D83	–	–	–	–	H69

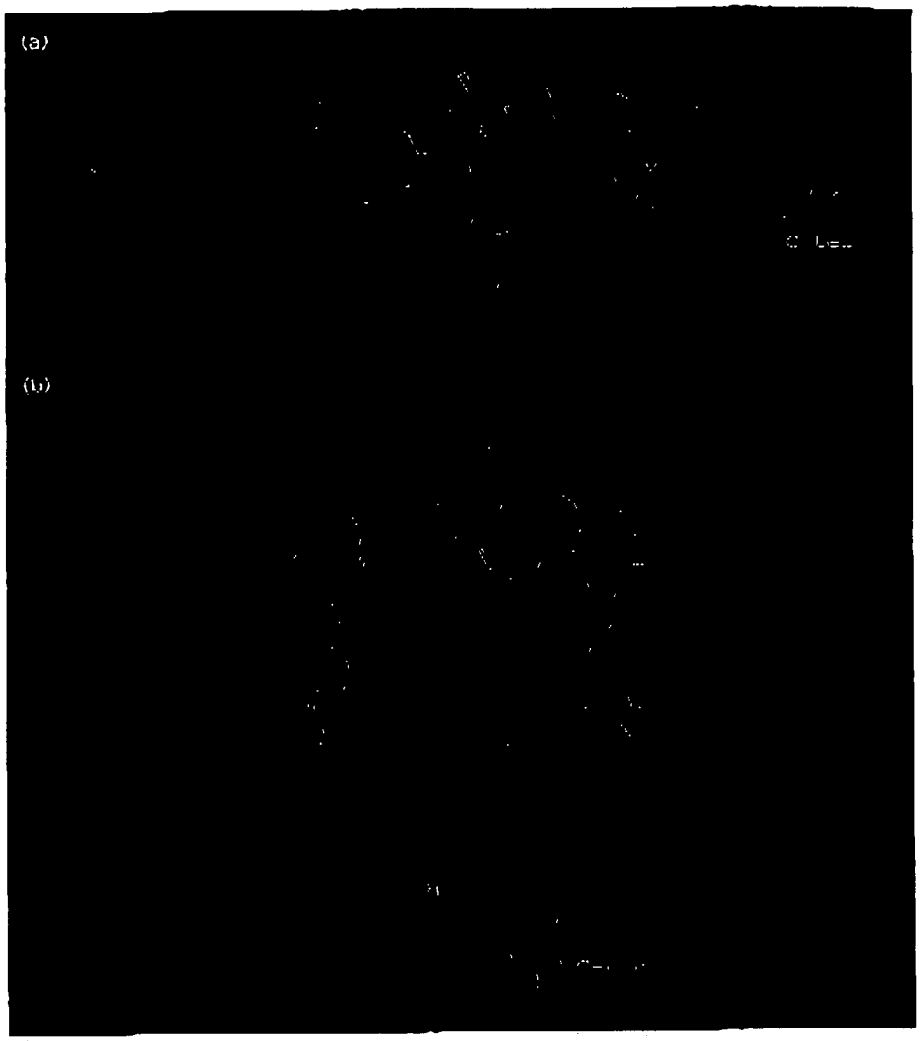
*Hydrogen bond and ion pairs between atoms from residues in monomer A with atoms from residues in monomer B (cut-off is 3.3 Å). Residues are identified by one letter code and sequence number. †For each residue in monomer A, van der Waals partners in monomer B are listed. The number in parentheses indicates the number of atoms in that residue within 4.0 Å of the corresponding residue in monomer A.

approximately 40-residue subdomain. The subdomain has the sequence Cys1–x_{10-15}–Cys2–x_2–Cys3 –x_2–Cys4–x_{8-11}–Cys5–x_{7-8}–Cys6, where x_{n-m} denotes n to m intervening amino acids. The subdomain is characterized by three disulphides: Cys1–Cys2, Cys3–Cys5 and Cys4–Cys6. This structural unit is exemplified by subdomains two (55–96) and three (98–137) of sTNF-R1 and has been described in detail [7,8]. However, subdomain one (15–52), has no intervening residues between Cys2 and Cys3. As a result, the orientation of the N-terminal S-shaped loop relative to the C-terminal S-shaped loop is quite different when compared with subdomains two and three (Figs 1,2). Subdomain four (139–166) is yet more divergent, as discussed above.

In accordance with Bazan [5], we now suggest that the extracellular domain of TNF-R1 is built up of three distinct modules, which we term A1, B2 and C2, rather than the 40-residue subdomain favoured in earlier reports [7,8]. A and B modules may contain either one or two disulfide bonds, thus giving rise to the designation A1, B2 etc. The A1 module is the N-terminal S-shaped loop found in all four subdomains. The B2 module is the C-terminal S-shaped loop found in subdomains one, two and three. The C2 module corresponds to the C terminus of the fourth subdomain, which is disordered in all but the orthorhombic crystals. Subdomains one, two and three are each a linear combination of an A1 and a B2 module. Subdomain four is a linear combination of an A1 and a C2 module. Figure 2 shows how a subdomain is decomposed into its constituent modules and Figure 5 shows the structure of the A1 and B2 modules. The modules are connected to each other by a varying number of intervening acids (0, 1 and 2). The modular composition of sTNF-R1 is shown in Figure 6.

The four A1 modules have the sequence Cys1–x_2–Gly–x_1–Tyr(Phe)–x^a_1–x^b_{4-9}–Cys2: we use superscripts to identify particular residues or regions within the structure. Each module has a structurally conserved core of eight amino acids (Cys1–x_2–Gly–x_1–Tyr–x^a_1 and Cys2) whose Cα atoms superimpose with an average root mean square (rms) deviation of 0.4 Å (Fig. 5a). This core includes the conserved aromatic residue highlighted by Banner *et al.* [8]. Structural variability is seen at the x^b_{4-9} loop (at the bottom of Fig. 5a). Each A1 module has a different sequence and number of amino acids in this loop. The B2 modules at residues 73–96 and 117–137 have sequence Cys3–x_2–Cys4–$x^c_{3/6}$–x^d_5–Cys5–x^e_4–Asp–Thr–X–Cys6 where n/m denotes n or m intervening

Figure 4



(a) The parallel dimer found at pH 7.5; this dimer does not occlude the TNF-binding site. (b) The antiparallel dimer found at pH 7.5 occludes the TNF-binding site. The color scheme is as in Figure 3. (Figure prepared using RASTER3D [31].)

residues. This gives 18 superimposable amino acids with a Cα rms deviation of 0.7 Å. The loop $x^{o}_{3/6}$ (top of Fig. 5b) is different in both sequence and in the number of amino acids; it was therefore excluded from the superposition calculation. The B2 module in subdomain one (residues 30–52) again has a different loop composition four residues) and structure at x^{c}. However, it also differs in that it has five rather than four residues at x^{c}. This results in only 14 superimposable amino acids with an average rms deviation of 0.8 Å for Cα atoms. The conserved core of amino acids includes the Asp–Thr–x–Cys6 motif highlighted previously [8].

It is notable that in both modules, structural divergence is associated with differing numbers of residues in a loop and that sequence divergence occurs in structurally conserved loops. For instance the glycine in the A1 module is replaced by asparagine in subdomain three. We therefore suggest, that it is the number, rather than the type, of residues that determines the structure of variable loop regions in the A1 and B2 modules. We have extended our analysis to the entire TNF-R superfamily (JHN and SRS, unpublished data) and have found that the vast majority of TNF-R1 homologues can be described as combinations of A and B modules.

Segmental flexibility: structural differences on pH change and LTα binding

The low pH form in combination with other structures of sTNF-R1 allows us to describe the changes in receptor conformation that occur in response to environment. Table 5 summarizes the results of the superpositions of the six sTNF-R1 structures. When the monomer is considered as a single unit, the rms deviations in Cα positions range from 1.1 to 2.8 Å. For monomers related by noncrystallographic symmetry (ncs), the rms deviation is 1.4 Å. Splitting the

Table 4

Non-bonded contacts between parallel dimers in tetragonal (pH 7.5) crystals of sTNF-R1.

Polar contacts*					van der Waals
Residue A	Atom A	Residue B	Atom B	Distance (Å)	(≤4.0Å)†
M11	E54				
Q17	Nε2	K65	O	3.3	T37(4), K35, G36(3), E54, V90(2), D91
G18					H34(5)
K19	Nζ	D49	Oδ2	3.1	D49(2)
T31	Oγ1	D49	Oδ1	2.7	H34(2), D49(3)
K32	O	H34	Nε2	2.8	H34(8)
C33					H34(2)
H34	Nε2	K32	O	2.8	G18(2), T31(2), K32(8), C33(2), H34(8), E64(3)
K35	O	Q17	Nε2	3.1	Q17(2), E64(3)
G36					Q17(4)
T37					Q17(3)
G47	O	D49	N	2.9	Q48(3), D49(4)
Q48	Nε2	Q48	Oε1	2.9	G47(4), D48(4), Q49(7)
D49	N	G47	O	2.9	K19(2), T31(4), G47(4), Q48(3),
	Oδ2	T31	Oγ1	2.6	D49(2)
E54					Q17
E64					H34(4), K35(2)
V90					Q17(2)
D91					Q17
H126					V136(2), L145
L127					Q133, V136
Q130					Q133(2)
Q133	Oε1	Q133	Nε2	2.9	L127, Q130(3), Q133(6)
	Nε2	Q133	Oε1	2.9	
V136					H126(2), L127, V136
C137					H126(2)
T138					L145
L145					H126(5), T138

*Hydrogen bond and ion pairs between atoms from residues in monomer A with atoms from residues in monomer B (cut-off is 3.3 Å). Residues are identified by the one letter code and sequence number.

†For each residue in monomer A, van der Waals partners in monomer B are listed. The number in parentheses indicates the number of atoms in that residue within 4.0 Å of the corresponding residue in monomer A.

monomers into the four subdomains reduces these values and splitting the structures into modules reduces them to ≤0.5 Å (i.e. more typical values for structural superposition). Thus it is the modules which are structurally conserved among the structures of sTNF-R1 rather than the subdomains. The high rms deviations for the monomer and subdomain superpositions are due to structural flexibility in the connections between modules. These connections are equally flexible whether they are within or between subdomains. For example, the ncs superposition of residues 30–70 (B2–A1 module combination with connection between subdomains) is 0.4 Å, approximately the same as residues 55–96 (A1–B2 module combination with connection within a subdomain). Splitting subdomain one into two modules yields a less dramatic drop in rms deviation, a consequence of the lack of intervening residues between the modules and hence decreased flexibility. The most pronounced flexibility is seen at Gly97, the connection between the second and third subdomains (Fig. 7). It is the segmental rigid-body movements of modules that underlie deviations from ncs in sTNF-R1.

Segmental flexibility allows sTNF-R1 to adapt its structure to changes in solvent conditions and LTα binding. These adaptations are most dramatically illustrated by superimposing all structures of sTNF-R1, but using only

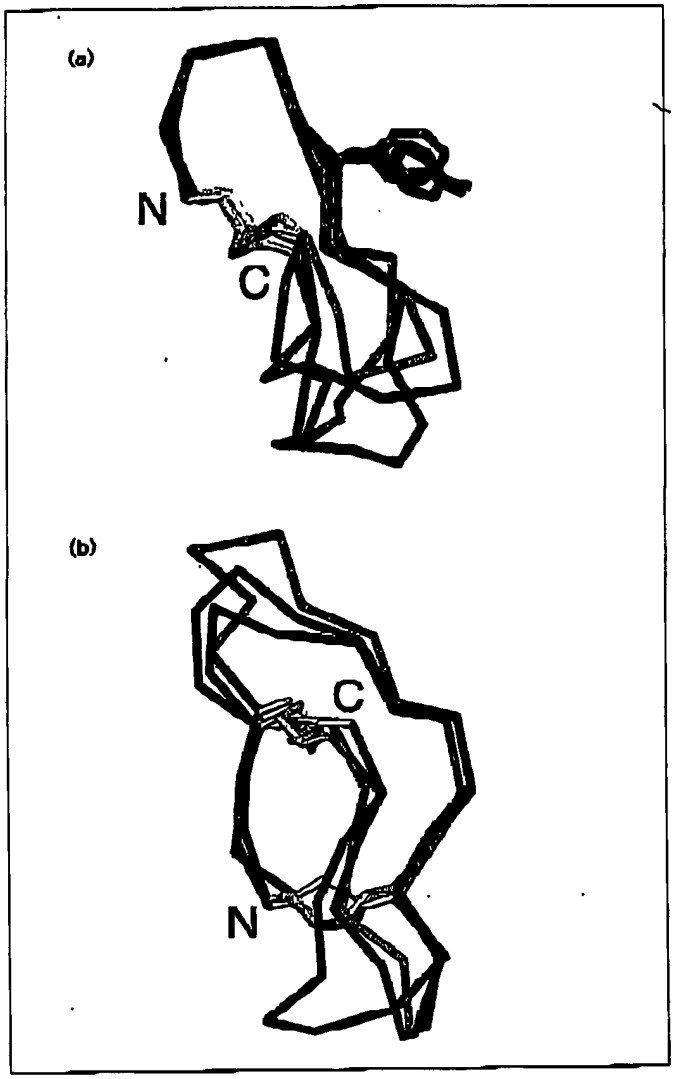
Table 5

Root mean square deviations (Å) in Cα superpositions.

Reference	Target molecules for superposition			
orthorhombic*	Orthorhombic†	Hexagonal	Tetragonal	LTα complex
Monomer	1.4	1.1	2.9	1.8
Subdomains	0.4	0.5	0.8	0.7
Modules	0.2	0.4	0.5	0.5

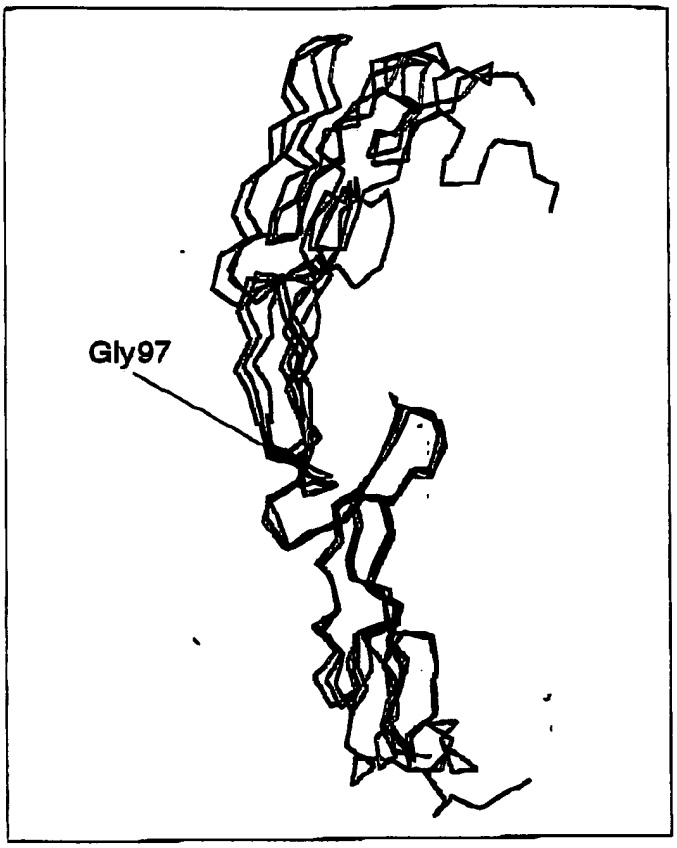
*Monomer A of the orthorhombic crystal form; †monomer B of the orthorhombic crystal form.

Figure 5



The modules which are structurally conserved within sTNF-R1. (a) An overlay of the four type A modules found in sTNF-R1: residues 15–29 (dark blue); residues 58–70 (grey); residues 98–114 (red); and residues 139–150 (light blue). The side chains of the conserved aromatic residues are shown, the cysteine residues are shown in yellow. (b) An overlay of the three type B modules found in sTNF-R1: residues 30–52 (blue); residues 79–96 (grey), and residues 117–137 (red). Cysteine residues are shown in yellow. Figure prepared using SETOR [32].

Figure 6



Superposition of sTNF-R1 monomers from the orthorhombic (low pH) crystal form (grey), tetragonal (pH 7.5) crystal form (red) and LTα complex (blue). The superpositions were calculated using only the Cα atoms of subdomain three. The segmental flexibility of the monomer is clear, with dramatic shifts in the N-terminal subdomains. The fourth subdomain of the orthorhombic crystal form is clearly positioned differently from the other two. (Figure prepared using SETOR [32].)

residues in subdomain three to calculate the superposition vector (Fig. 7). This partially mimics the biological environment where the C terminus is anchored to the membrane. This superposition results in a difference of over 15 Å in the position of N terminus between the high [7] and low pH forms of the receptor. The LTα-bound form of sTNF-R1 [8] lies between these extremes. As a result, the low pH monomer is less curved with a distance between the first and tenth disulphide of 66 Å compared with 62 Å at pH 7.5 and 64 Å in the LTα-complex form. The fourth subdomain adopts a different orientation relative to third subdomain in the low pH form when compared with the other structures. This shift in position is over 4.0 Å and is clearly visible in Figure 7. We interpret the movement of the fourth subdomain as being a consequence of its participation in dimer contacts and the hydrogen bonds that it forms to the third subdomain; Asn148 to Asn116 and Asn148 to Ser118. Both this dimer and these additional hydrogen bonds are unique to the low pH crystals. In the orthorhombic structure the ninth disulphide (Cys129–Cys137) adopts unusual $\chi 1$ values of 180°/180°, in contrast to the pH 7.5 and LTα complex structures where the corresponding values are 60°/60°. The conformation of the ninth disulphide in the hexagonal form is ambiguous. It seems likely that this change in

Table 6

Crystal parameters, data collection and refinement statistics.

	$P2_12_12_1$	$P6_122$
Unique reflections	37300	13334
Completeness of data		
entire resolution range $I>0\sigma I$ ($>3\sigma I$)	11.0–1.85 Å 97% (91%)	42.0–1.87 Å 97% (88%)
highest resolution shell $I>0\sigma I$ ($>3\sigma I$)	1.93–1.85 Å 77% (64%)	1.95–1.87 Å 91% (68%)
$R_{merge}(I)$ (%)*	5.1	5.5
Average data redundancy	5	6
Refinement		
Resolution range (Å)	8–1.85	42–1.87
R free (%)†	24.3	27.8
R factor (%)	20.5	23.9
Bond rms deviation (Å)‡	0.009	0.009
Angle rms deviation (Å)‡	1.686	1.790
Ramachandran core/addtnl (%)§	90.2/9.8	85.0/12.0
Protein mean B ($Å^2$)	18	35
Solvent mean B ($Å^2$)	30	43
B-factor bond rms deviation (Å)#	2.0	3.4
Protein atoms	3080	1255
Solvent atoms	403	120

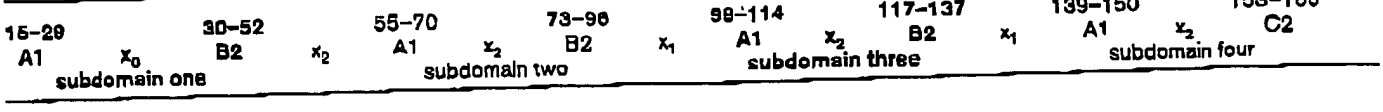
*$R_{merge(I)}=\Sigma_{hkl}\Sigma_i|I_i-I(hkl)|/\Sigma_{hkl}\Sigma_i I_i(hkl)$; †R free is calculated on 10% of data excluded from gradient calculation during refinement; ‡rms deviation from Engh and Huber ideal values [28]; §core and additionally allowed regions as defined by PROCHECK [29]. In $P6_122$, one residue is in the generously allowed region and two are in disallowed regions; all three residues are in poorly defined regions of electron density. #Rms deviations on B factors between bonded atoms, calculated with MOLEMAN (GJ Kleywegt, unpublished program); atoms with zero occupancy are excluded from all B factor calculations.

disulphide conformation is correlated with the movement of the fourth subdomain. We note that constructing a model of the parallel dimer form seen at pH 7.5 (Fig. 4a) with the complete monomer leads to interpenetration of subdomain four, strongly suggesting that the position of this subdomain is flexible. Comparing the two unliganded structures, shifts in local regions of the main chain (up to 1.0 Å) occur at regions that are involved in dimer or crystal contacts, particularly at Pro46, Ser74 and Val90. In the LTα complex, the main chain at Asn25 is shifted relative to both unliganded structures. However, this may reflect the fact that in the LTα complex structure, this residue was glycosylated. The orientation of side chains at Ser63, 72 and 108 change on LTα binding in order to make hydrogen bonds with the cytokine. A number of other differences in side-chain conformation are seen, but these are either found in flexible regions or differ between the various unliganded receptor structures.

Biological implications

Tumor necrosis factor (TNF) is responsible for dramatic changes in cellular metabolism. TNF exerts its affects through two receptors, TNF-R1 and TNF-R2. Both receptors belong to a superfamily that includes FAS, low affinity nerve growth factor (NGF) receptor and CD40. We report the crystal structure of the extracellular domain of the TNF-R1 receptor (sTNF-R1), crystallized at low pH and at high ionic strength; the receptor exists as a dimer in two crystal forms. The dimer is formed by an antiparallel association between the monomers at a dimerization interface that overlaps with the LTα-binding surface. The interface is considerably more extensive than that involved in complex formation with LTα. We do not claim that acidic conditions are specifically responsible for the formation of the sTNF-R1 dimers observed in the orthorhombic crystals. However, such dimers could form when TNF-bound receptors are internalized and enter the acidic compartment of the endosome. Dimer formation would displace bound TNF and could allow the receptors to be recycled. We have previously shown that sTNF-R1 crystallized at pH 7.5 from methylpentandiol forms both an antiparallel and a parallel dimer, which are distinct from the dimer described here. We suggested possible roles for 'high pH' dimers in signal regulation and receptor internalization. No direct evidence exists to support a dimeric receptor species in solution. Nevertheless, the entropy penalty for dimerization on the membrane would be significantly

Figure 7



less than for dimer formation in dilute solution. Further studies of the aggregation properties of the receptor *in vivo* will be required to assess the true biological relevance of ligand-independent receptor aggregation.

Three of the four ~40 amino-acid subdomains that comprise the extracellular domain of TNF-R1 are composed of a linear combination of an A1 module and B2 module. The A1 module is the S-shaped double hairpin structure that forms the N-terminal half of the subdomain. The B2 module is a similar but distinct S-shaped structure that forms the C-terminal half of the subdomain. The fourth subdomain, which had been largely disordered in all previous studies, consists of an A1 module followed by an atypical C2 module. The C2 differs in topology and disulphide connectivity from both the A1 and B2 modules. Elsewhere, we demonstrate that all members of the TNF-R superfamily are combinations of modules. In sTNF-R1 it is the modules rather than the subdomains that are structurally conserved. The connections between the modules are flexible: consequently, sTNF-R1 exhibits segmental rigid-body movements in response to pH changes and LTα binding. Modules may correspond to functional as well as structural units. The compartmentalization of binding activity within small, discrete structural units has important implications both for the evolution of this family of receptors, and for the design of receptor antagonists.

Materials and methods

Crystallization and data collection

Recombinant sTNF-R1 was expressed and purified as previously described [21]. Although the protein used was refolded from inclusion bodies, kinetic and thermodynamic measurements indicate that it is identical to the protein found in serum and urine. Crystals in space group $P2_12_12_1$, with unit-cell parameters a=78.6 Å, b=83.1 Å, c=67.9 Å were obtained at pH 8.7 with $MgSO_4$ as the precipitant, as described [21]. More recent batches of protein have produced a second crystal form under exactly the same conditions as used for the $P2_12_12_1$ form. This form occurs as hexagonal bipyramids in spacegroup $P6_122$ with unit-cell parameters a=b=69.5 Å, c=112.8 Å. The percentage of each form varies from 0–100% within any crystallization experiment, although $P2_12_12_1$ predominates. We have as yet found no systematic way of selecting one form over the other. We cannot identify any changes in the protein from one batch to the next and we have not modified our purification or crystallization protocols. All batches of the protein crystallize at pH 7.5 with MPD [21]. Although we think it is highly unlikely, we cannot exclude the possibility of the presence of folding isomers (at the C terminus).

Data to 1.85 Å for the $P2_12_12_1$ crystal form were collected from a single frozen crystal mounted in a nylon loop at Station PX9.2 at the Daresbury synchrotron, with a wavelength of 0.892 Å on a Mar image plate. The crystal was cryoprotected with 12% glycerol prior to data collection. The intensity data were processed and merged with DENZO and SCALEPACK [22]. Further data reduction was carried out with the CCP4 package [23]. Data reduction statistics are shown in Table 6. The first data set on the $P6_122$ form was recorded with a room temperature crystal using the Xuong-Hamlin area detector and a copper rotating anode source. The crystal was radiation sensitive and only a 60% complete 2.8 Å data set was recorded. A second data set on a cryoprotected (12% glycerol) $P6_122$ crystal was collected to 1.87 Å using an R-AXIS II with a copper rotating anode source. The intensity data were processed and merged with DENZO and SCALEPACK [22]. Data reduction statistics for the frozen $P6_122$ crystal are shown in Table 6.

Structure solution and refinement

The $P2_12_12_1$ crystal form had not proven tractable to isomorphous replacement. Molecular replacement using sTNF-R1 dimers or monomers or partial structures thereof from the $P4_12_12$ structure [7] and the LTα complex [8] failed to yield a solution. The room temperature data set on the $P6_122$ crystal form then came to hand. With a monomer in the asymmetric unit this crystal form was readily solvable with CCP4 AMORE [24] using a monomer from the $P4_12_12$ structure [7] as the search model. Despite the poor quality of the data this structure was refined to an R factor of 25%, and R free of 38%. The structure was ordered only from residues 15–137 and electron density was weak and noisy for residues 110–137. This partially refined partial structure was used as a model for a further molecular replacement attempt on the $P2_12_12_1$ structure. This search model produced a single weak solution with CCP4 AMORE, but only when normalised structure factors (Es) were employed. This single solution readily located the second monomer in a phased translation search. The two monomers were not related by any peak in the Patterson self-rotation map.

The $P2_12_12_1$ structure was refined using X-PLOR [25] and manual intervention was carried out using O [26]. Electron-density maps were calculated with data from 11–1.85 Å using SIGMAA-weighted coefficients [27]. The model was refined against 90% of the measured data between 8–1.85 Å using the Engh and Huber stereochemical dictionary [28]. The remaining 10% of measured data between 8 and 1.85 Å were excluded from all refinement calculations to monitor progress of refinement. No sigma cut-off was applied to the data. Weak noncrystallographic restraints were imposed on both positional and B-factor refinement, but regions of crystal contact were excluded. The initial model (R=55%, R free=64%) had to be extensively rebuilt, particularly residues 97–137. It was immediately obvious in the first F_o–F_c maps that a large C-terminal portion of the molecule was present. Residues 138–172 for one monomer and 138–168 for the other were built into density. Water molecules were included in batches provided they satisfied four criteria: they corresponded to a peak at $\geq 3.5\sigma$ (0.26e/Å^{-3}) in the F_o–F_c map; they formed potential hydrogen bonds with reasonable stereochemistry; they reappear in at least 1σ in subsequent $2F_o$–F_c maps (0.26e/Å^{-3}); and adding the batch of water yielded a reduction in R free. Once convergence was achieved, the model was then refined against all measured data from 8 to 1.85 Å for 20 cycles of Powell minimization. Statistics on the final model are shown in Table 6.

To confirm the difference in the conformation of disulphide nine between the orthorhombic and tetragonal structures, we calculated simulated-annealing SIGMAA omit maps for both structures. We also rebuilt each like the other and attempted to refine these models. However, difference electron density strongly suggested that the original conformers were correct in each case. We are convinced that this is a real difference in the structures.

The 1.87 Å data set on the $P6_122$ crystal was collected after the $P2_12_12_1$ structure had been fully refined. The hexagonal structure was redetermined using CCP4 AMORE with a monomer from the orthorhombic structure. The C-terminal 34 residues had to be removed from the search model to yield the correct solution. This structure was refined using data (no sigma cut-off) from 42–1.87 Å with the X-PLOR bulk solvent correction [25]. Statistics on the final model are shown in Table 6. The $P6_122$ structure is extremely disordered in some residues: only residues 14–120 and 127–135 are ordered. Residues 13, 121–127 and 135–139 were built into weak and ambiguous density on the basis of prior sTNF-R1 structures. Residues 11, 12 and 140–172 appear to be completely disordered, consistent with our observation that including this region in the search model gives an incorrect solution. We feel that

our failure to accurately model these disordered regions is responsible for the relatively high R free of 27.8%.

Protein stereochemistry was assessed with X-PLOR [26] and PROCHECK [29]. Programs from the Uppsala suite were used to measure differences in dihedral and side-chain angles between monomers. Superpositions and buried surface area calculations were carried out using the CCP4 package [23].

Accession numbers

The coordinates of the $P2_12_12_1$ dimer and the data set have been deposited with the Brookhaven Protein Data Bank [30] (entry codes 1EXT and R1EXTSF).

Acknowledgements

We thank Mike Eck, Barbara Brandhuber and Lynn Rodseth for early contributions to the project; Albert Berghuis, Sean McSweeny, Peter Lindley and Elizabeth Duke for help with data collection at Daresbury; Bryan Sutton for skilled assistance with figures. We are grateful to John Tesmer for his assistance in measuring and processing the high resolution P6$_1$22 data set. We thank Werner Lesslauer and Dave Banner (Hoffmann La Roche) for prerelease of the LTα–sTNF-R1 complex coordinates. We thank the reviewers and editors for helpful comments. JHN acknowledges the Wellcome Trust (043588/Z/95/Z/RF/PK), the University of St. Andrews and the BBSRC (ref 49/B06675) for support, and the Daresbury Laboratory for data collection facilities.

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Pflügers Arch - Eur J Physiol (2000) 439 [Suppl]: R113-R115

Early events in TNFa - p55 receptor interactions – experiments with TNF dimers

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Abstract The first essential step in TNF signal transduction is believed to be clustering of the membrane bound receptors around the trimeric TNF molecule. To check if one receptor binding site would be enough to trigger the signal, we tried to prepare several types of TNF dimer. For this purpose, two TNF analogs bearing different cysteine mutations at the inner subunit binding surfaces were designed, expressed in *E. coli* and prepared in pure form. By mixing equimolar quantities of these analogs under appropriate conditions, two different types of dimer were prepared. The first, Dim/S2, proved to be composed mainly of a disulfide-linked dimer, which was still capable of trapping the third subunit of either of the precursor analogs, thus showing relatively high residual cytotoxicity. To avoid trimeric structures, Dim/S2 was further transformed into Dim/Iaa2 by alkylation of -SH groups of the newly introduced cysteines, allowing binding of only two TNF subunits through native contact surfaces. These dimers showed substantially reduced cytotoxicity on the L929 cell line. In addition, it appears that Dim/Iaa2 is able to competitively inhibit cytotoxicity of native TNF, as assessed on the L-M cell line.

Key words TNF-α · analog · dimer · trimer · cytotoxicity cysteine · disulfide bond · histidine · IMAC

Introduction

Tumour necrosis factor alpha (TNF) was originally identified by its anti-tumour activity against certain tumours implanted in mice and cytotoxic action on tumour cells *in vitro* [3]. Though the exact mechanism of its cytotoxicity on malignant cells is still largely unknown, today TNF anti-tumour activity is usually ascribed to a concerted action of direct cytotoxicity on tumour cells, action on the endothelium of the neo-capillary system and immune response activation. Like numerous pleiotropic effects, which TNF executes in inflammatory and cellular immune responses, its direct cytotoxicity is also mediated by TNF transmembrane receptors. In the recent years, several models for the interaction of TNF and p55 receptor have been proposed, among which trimerisation, expanding-network and molecular switch hypotheses, are the most popular [2]. Clustering of receptors is believed to be a triggering step for signal transduction. Being generally accepted that TNF is biologically active only in the form of trimers [9], in all the above mentioned models, TNF enters as a compact trimer with three equivalent receptor binding sites in the broader part of the bell-shaped TNF molecule. Since a single binding site involves specific amino acids from two adjacent TNF subunits, stretching on both sides of the subunit contact in trimeric TNF [10], a dimeric TNF bearing only one receptor binding site should, according to the above mechanisms, be biologically inactive. On the other hand, it might be assumed that one receptor binding site would be sufficient to bind to each membrane receptor, thus preventing the binding of natural trimeric TNF. One direct approach to verify these assumptions would be application of TNF trimers having one, two or all three active sites blocked. Experimentally this would be very difficult, so we used a different approach and tried to prepare dimers containing only a single native receptor-binding site. Design and preparation of two TNF analogs, which served as precursors for dimers, preparation of two kinds of dimer, some *in vitro* cytotoxicity measurements and preliminary inhibitory experiments, are the subject of this contribution.

Materials and methods

3D structure of human TNF-α was obtained from the Brookhaven Protein Data Bank and analysed by ISIGHT II (Biosym Technologies Inc.) program and IRIS graphical terminal. Synthetic human TNF-α gene (in BBG4 plasmid) with codons, optimised for expression in *E. coli*, was purchased from British Biotechnology.

Preparation of TNF analogs (precursors for dimers)

For preparation of precursor TNF-α analogs LK-817 (Cys95His107His108) and LK-818 (Cys148His107His108), expression system *E. coli* AD494/pCYTEXP1 ΔcI with appropriate TNF-α mutant genes was used.

Mutations were introduced by oligonucleotide-directed mutagenesis on ssDNA according to Kunkel. Fermentation was done in 7 litre CHEMAP fermentor at 25 °C, 600 rpm and aeration of 1 vvm. After centrifugation at 5000 rpm, the cell pellet was resuspended in 50 mM TRIS/HCl, 30 mM NaCl, pH 8.0 and disrupted by sonication. In the crude homogenate, nucleic acids were removed by polyethyleneimine precipitation. Supernatant containing soluble TNF analog was precipitated by 65% saturated ammonium sulphate. Chromatographic separations were performed on FPLC chromatographic system (Pharmacia) at a flow rate of 1 ml/min. Both analogs were purified by single step chromatographic procedures on Cu^{++} loaded Chelating Superose column (Pharmacia). Binding of histidine containing TNF analogs was achieved in 0.02 M K-phosphate, 0.2 M NaCl, pH 7.1 buffer, and elution was performed by increasing gradient of 50 mM imidazole in the same buffer using two different elution profiles.

Preparation of dimers

Both types of dimer were prepared by mixing equimolar quantities of precursor analogs, LK-817 and LK-818, with excess of sulfitolysis reagent in 100 mM TRIS/HCl, 10% DMSO, 0.2% Hecameg, pH 9.0. After removal of sulfitolysis reagent by ultrafiltration or dialysis, a 10-fold molar excess of DTT was added. Oxidation during extensive dialysis against 0.02 M phosphate buffer with 0.2 M NaCl, 5% DMSO, 0.2% Hecameg resulted into disulfide-linked Dim/S2. Procedure for preparation of S-alkylated dimer Dim/Iaa2 was essentially the same, with an additional alkylating step by ICH_2CONH_2, following a DTT reduction step. Protein concentrations were assayed by Bradford procedure. SDS-PAGE was performed under non-reducing conditions and protein bands were visualised by Coomassie R-350 staining.

Biological activity of precursor analogs and dimeric species prepared was determined by an *in vitro* cytotoxicity assay on mouse fibroblast L929 cell line [5]. Preliminary inhibition studies were performed on less sensitive L-M cell line [6].

Results

Oligomeric forms of precursor TNF analogs. Precursor analogs LK-817 and LK-818 (Fig 1) were designed so that a pair of histidines was introduced into exposed flexible loop of each TNF subunit, thus allowing simple purification by Immobilised Metal Affinity Chromatography (IMAC). Since elution times in this type of chromatography are well correlated with the number of histidines [7], IMAC additionally offers an information about oligomeric state of the molecule. Using IMAC (results not shown) we found, that precursor TNF analogs differed significantly in chromatographic behaviour, LK-817 being more efficiently bound than LK-818. This indicates that LK-817 exists in solution entirely in the trimeric form. On the contrary, LK-818 behaves like a dimeric species. The same picture was confirmed by SDS-PAGE under non-reducing conditions (Fig 2). Subunits in LK-817 trimer are bound similarly as in native TNF trimer, indicating that the newly introduced Cys95 does not disturb the local structure. This is also confirmed by its relatively high biological activity (Table 1). No disulfide-linked dimers are detectable. Interestingly, in the case of LK-818 only disulfide-linked dimers are present. Due to just one cysteine (Cys148) mutation, only non-native dimers are possible, which is also confirmed by significantly reduced cytotoxicity (Table 1). Obviously newly introduced Cys148 residues are sufficiently exposed and reactive to form a disulfide bond.

Dimers. By mixing equimolar amounts of TNF precursor analogs as described above, two different types of dimer

TNF-α analogs	Graphic representation[1]
LK-817	● -SH
LK-818	— -S-S-
Dim/S2	
Dim/Iaa2	▭-S-CH_2CONH_2

[1] *cross-section of TNF putative oligomeric forms perpendicular to 3-fold axis of symmetry*

Fig 1 Newly prepared TNF-α analogs and some dimeric forms

Table 1 Cytotoxic activities of new analogs and dimers

TNF-α analogs	Cytotoxicity[2] (U/mg)	% activity of TNF standard 87/650 (= $4*10^7$ U/mg)
LK-817	$9.7*10^6$	24 %
LK-818	$5.6*10^5$	1.4 %
Dim/S2	$2.4*10^7$	60%
Dim/Iaa2	$1.1*10^6$	2.8 %

[2] *determined by standard bioassay on mouse L929 cells*

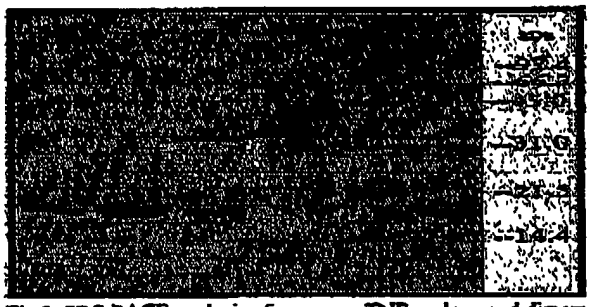

Fig 2 SDS-PAGE analysis of precursor TNF analogs and dimers under non-reducing conditions: 1 TNF-α standard; 2 LK-817; 3 Dim/S2; 4 LK-818; 5 Low MW standard (BioRad); 6 Dim/Iaa2

Dim/S2 and Dim/Iaa2 were prepared. In the case of Dim/S2, the combination of precursor analogs bearing a matching pair of cysteine mutations, resulted in the formation of a disulfide bond between subunits of different analogs. This covalently connected the native binding surfaces of TNF subunits. SDS-PAGE (Fig 2) revealed that Dim/S2 was composed mainly of a disulfide-linked dimer, exhibiting a slightly lower apparent molecular weight than the precursor analog LK-818, which formed non-naturally linked dimers. However, in Dim/S2 a third subunit of either of the precursor analogs was additionally trapped (Fig 1) into the cavity of the disulfide linked dimer, resulting in hybrid trimers, which retained relatively high biological activity (Table 1). While we were not able to remove the third subunit during the preparation of Dim/S2, we tried to prepare another type of dimers - Dim/Iaa2 - by alkylating

the thiol groups of cysteines using iodoacetamide.

SDS-PAGE analysis (Fig 2) revealed that the Dim/Iaa2 was not completely alkylated and retained a low amount of disulfide-linked dimers. We noticed that Dim/Iaa2 preparation exhibited significantly reduced solubility in comparison to native (trimeric) TNF although DMSO and Hecameg were added in order to improve it. Despite this, concentrations higher than 0.1 mg/ml and prolonged storage at + 4 °C caused precipitation. On IMAC, Dim/Iaa2 was eluted essentially as a dimeric species with 4 exposed histidines (data not shown). When measuring cytotoxicity on L929 cells Dim/Iaa2 displayed only a few percent of the full cytotoxic activity of trimeric TNF (Table 1). All these properties suggested Dim/Iaa2 as a good candidate for testing early interactions between TNF and its receptors.

In an attempt to show possible antagonistic action of Dim/Iaa2 against native trimeric TNF, no apparent inhibition could be detected when the highly sensitive L929 cell line was used. Presumably minor residual impurities (trimeric TNF) still present in the Dim/Iaa2, made the preparation still cytotoxic itself when it was used in excess. However, when a less sensitive L-M cell line was used for these experiments an apparent inhibitory action was recognised. Preliminary experiments showed that a 13-fold molar excess of Dim/Iaa2 inhibited trimeric TNF cytotoxicity by up to 50%.

Discussion

Oligomeric forms of TNF have been studied by different approaches [1,8] leading to generally accepted conclusions that trimeric TNF is the only form responsible for full induction of cytotoxic signal through TNF receptors. It is also proposed that low *in vivo* stability is at least partially due to dissociation of TNF to monomers which are more susceptible to proteolytic attack. So far, dimers were not considered to be involved or important *in vivo* although one should expect their presence if a dissociation mechanism is accepted as a probable event [4]. It is obvious that dimers of native TNF subunits are not easy to prepare, due to equilibrium lying far on the side of trimers, which are also believed to be the biologically active forms [9]. Actually, there are no reports on successful preparation of dimers arising from native TNF trimers. Also in our experiments, using a special approach, it was not that straightforward to prepare a dimeric TNF species in soluble form. Although reports on preparation of monomeric TNF exist in the literature [1,8], it appears that, as expected, exposure of hydrophobic interior surfaces makes the molecule very prone to precipitation and denaturation. In native TNF trimer, amino acid residues from two adjacent subunits constitute a single receptor binding site [10] thus resulting in a total of three equivalent receptor binding sites per TNF trimer. In non-naturally formed TNF dimers, as artificially achieved in the case of analog LK-818, linked through newly introduced cysteines, no complete receptor binding site is available. Consequently this molecule should not trigger a cytotoxic signal and this was confirmed experimentally. A dimeric form of TNF (Dim/Iaa2) was prepared in such a way that it bears only one complete and active receptor binding site. Its cytotoxicity was significantly reduced, indicating that the single receptor binding site is not enough for efficient transfer of cytotoxic signal via TNF receptors. Therefore, our results provide additional experimental evidence for the hypothesis, that clustering of receptors must be a key event in the early stages of TNF - p55 receptor interaction and signalling. On the other hand, preliminary results of Dim/Iaa2 acting as a competitive inhibitor against native trimeric TNF, show that one receptor binding site is still enough to bind to membrane TNF receptors, and thus interfere with native TNF binding and signal transduction.

To conclude, in this preliminary study we wished to show that stable dimers of TNF subunits can be prepared and studied *in vitro* in order to obtain additional information on early TNF - receptor interactions. Our results confirm that dimers do not efficiently trigger the cytotoxic signal and also that excess of dimers can at least partially block the cytotoxic activity of native TNF trimers in L-M cells. Additional studies with thoroughly purified non-cytotoxic preparations are needed to draw conclusions on the possible roles of dimers in natural environments.

Acknowledgements This work was financially supported by Ministry of Science and Technology of Slovenia and Pharmaceutical and Chemical Company Lek d.d. We thank Mrs. J. Lenarčič and Mrs. A. Jesenko for their excellent technical assistance.

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